BREEDING MELONS FOR RESISTANCE TO VIRAL AND FUNGAL DISEASES. EXPLOITING THE MULTI-RESISTANT ACCESSION TGR-1551

Ph.D. dissertation by

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List of Abbreviations

PLANT VIRUSES		
BPYV beet pseudo-yellows virus	**CABYV** cucurbit aphid-borne yellows virus	**CCYV** Cucurbit chlorotic yellow virus
CGMMV cucumber green mottle mosaic virus	**CMV** cucumber mosaic virus	**CVYV** cucumber vein yellowing virus
CYSDV cucurbit yellow stunting disorder virus	**MeCMV** melon chlorotic mosaic virus	**MNSV** melon necrotic spot virus
MWMV Moroccan watermelon mosaic virus	**PPV** plum pox virus	**PRSV** papaya ring spot virus
SqLCV squasg leaf curl virus	**TMV** tomato mosaic vius	**ToLCNDV** tomato leaf curl New Delhi virus
WmCSV watermelon chlorotic stunt virus	**WMV** watermelon mosaic virus	**ZGMMV** zucchini green mottle mosaic virus
ZYMV zucchini yellow mosaic virus		

ABA abscisic acid
AFLPs Amplified Fragment Length Polymorphisms
AGAL α-galactosidases
amiRNAs artificial microRNAs
AUX auxins
GBS genotyping by sequencing
GCs gene clusters
gf green flesh
Go Golovinomyces orontii
hpi hours post-inoculation

BC Backcross
Bg7S basic 7S globulin-like protein
bHLH basic helix-loop-helix

BO `Bola de Oro ´

bp base pairs
BR brassinosteroids
bZIP basic leucine zipper
C4H cinnamate 4-hydroxylase
CAPs Cleaved amplified polymorphic sequence
CC-NLR coiled-coil containing nucleotide-binding leucine-rich repeat receptors
CDPK calcium-dependent protein kinase
Chr chromosome
CIM composite interval mapping
CP coat protein
CNR cell number regulator

CPM Cucurbits powdery mildew

CSR cell size regulator
CYP78A cytochrome P450
DEGs differentially expressed genes

DHL double haploid lines

dpi days post-inoculation
E environement
ecoTILLING Eco-type TILLING
EM groups `emerging´groups
ETI effector-triggered immunity
ET ethylene
ETI effector-triggered immunity

FD fruit diameter

FL fruit length
FS fruit shape
FW fruit weight
G genotype

Px *Podosphaera xanthii*
QTL Quantitative trait locus
RAPDs Random Amplified Polymorphic DNA
RdRp RNA-dependent RNA polymerase
REF4 reduced epidermal fluorescence 4
HR hypersensitive response
HRM high resolution melting
HSP heat shock proteins
Hsp70h heat shock protein 70 family homolog
ILs isogenic lines
indels insertions or deletions
INV invertases
JA jasmonate

KASP competitive allele-specific PCR

KEGG Kyoto Encyclopedia of Genes and Genomes
lncRNAs long non-coding RNAs
log2FC log2 fold change
MAPK mitogen-activated protein kinase
MAS marker-assisted selection
Mb mega base
MED33A RNA polymerase II transcription subunit 33A
MLO mildew resistance locus O
MYB myeloblastosis related
NAC no apical meristem
NBS-LRR nucleotide-binding site-leucine-rich
NGS new generation sequencing
NHP N-hydroxy-pipecolic acid
NILs nearly isogenic lines
OFP Ovate familiy proteins
ORF open reading frame
PA piperonylic acid
PAL1 phenylalanine-ammonia lyase 1
PAMPs pathogen associated molecular patterns
PPP phenylpropanoid pathway
PR pathogenesis related
PRR pattern recognition receptor
PS `Piel de Sapo´

RFLPs Restriction fragment length polymorphism
RFR1 REF4-related 1
RIL recombinant inbred lines
RIN RNA Integrity Number
RLKs receptor-like kinases
RNAPII RNA polymerase II
ROS reactive oxygen species
SAR systemic acquired resistance
SA salicylic acid
SMRT single-molecule real-time
SNPs single nucleotide polymorphism
SPS sucrose phosphate synthase
SS sucrose synthase
SSRs Simple sequence repeats
STKs serine/threonine-protein kinases
TCA citric acid cycle
TE transposable elements
TFs transcription factors
TIR-NBS-LRR
TILLING Target Induced Local Lesions in Genoms
TMM trimmed mean algorithm
TRM TONNEU1 Recruiting Motif
Trxs thioredoxin-like protein
UPS ubiquitin proteasome system
***Vat* gene** Virus aphid Transmission
wf white flesh
WGCNA weighted gene co-expression network analysis
wpi weeks post-inoculation
XEG xyloglucan-specific endo-β-1,4-glucanase
VvHT5 *Vitis vinifera* Hexose Transporter 5
VcwINV *Vitis vinifera* Cell Wall Invertase
VvHT5 *Vitis vinifera* Hexose Transporter 5

Abstract

Cucurbits represent the second most important horticultural family worldwide, second only the Solanaceae family. Traditionally, their cultivation has been concentrated in temperate regions across the globe. However, climate change conditions, international trade, and intensive agricultural practices are contributing to the emergence of new viral and fungal diseases in regions where they were previously absent. In this regard, it is crucial to regularly monitor major production areas to detect emerging viruses and fungi specific to each region. This monitoring allows for the adaptation of breeding programs to the unique goals of each area.

In the case of melon (*Cucumis melo*), it exists significant intraspecific variability that can serve as a source of resistance alleles against these pathogens. However, sources of resistance are often found within wild germplasm, typically originating from Africa or Asia, and characterized by limited domestication. To better utilize these resistant accessions, a study of the genetic control of desirable traits is necessary. This study aims to locate regions associated with resistance and design molecular markers linked to these regions. Such an approach streamlines breeding programs focused on introgressing resistance traits while preserving the genetic background of the desired varieties.

During the summer campaigns of 2019 and 2020, this doctoral thesis conducted a study on the incidence and genetic diversity of nine viral species potentially affecting cucurbit cultivation in southeastern Spain. It was observed that viruses transmitted by aphids were more prevalent than those transmitted by whiteflies. Within the first group, the presence of watermelon mosaic virus (WMV), cucurbits aphid borne yellows virus (CABYV), and cucumber mosaic virus (CMV) stood out, as they were detected in all the studied areas and crops, often in mixed infections. Moroccan watermelon mosaic virus (MWMV) and tomato leaf curl New Delhi virus (ToLCNDV) were also detected in some areas but with lower infection percentages, typically in mixed infections with WMV. Phylogenetic analyses of the found isolates have identified seven new molecular profiles of WMV and recombinant CMV isolates, which is consistent with results from other countries, highlighting the extensive variability of these pathogens.

Wild melon accessions preserved in various germplasm banks represent a valuable resource for breeding programs against biotic stresses. The African accession TGR-1551 has been previously described as resistant to WMV, CYSDV (cucurbit yellow stunting disorder virus), CABYV, and the fungus *Podosphaera xanthii* (*Px*, races 1, 2, and 5), which causes powdery mildew in melons. Additionally, it is tolerant to

whiteflies (*Bemisia tabaci*) and carries the *Vat* gene (*Virus Aphid Transmission*), limiting virus transmission by aphids. Therefore, this accession constitutes as an excellent source of resistance alleles, and its use, as a single donor parent, can expedite breeding programs.

Within the scope of this doctoral thesis, through the development of segregating mapping populations and the utilization of high-throughput genotyping technologies, the QTLs associated with CYSDV resistance from this accession have been mapped. In the case of CYSDV resistance, two QTLs have been detected on chromosome 5. The first of these, with major effects and dominant inheritance, is associated with symptom development. The second QTL, with minor effects and also dominant inheritance, does not confer resistance by itself and is linked to viral load during infection. A similar strategy was employed to map and narrow down the QTLs for resistance against *Px*. In this case, it involves a dominant-recessive epistasis, with the recessive gene located on chromosome 12 and the dominant gene on chromosome 5, specifically in the same region where the major CYSDV resistance QTL is located.

Regarding resistance against WMV, previous studies conducted by the research group indicated that it was controlled by a recessive gene on chromosome 11 and a minor QTL with modifying effects on chromosome 5. To gain deeper insights into the molecular mechanisms underlying this resistance, a transcriptomic analysis using RNA-seq was performed. This assay compared the differential response of a resistant line derived from TGR-1551 and a susceptible line to WMV infection. The results revealed a high degree of transcriptomic remodeling in the resistant line, with the activation of various defense mechanisms against pathogens, which contrasts with the recessive nature of the resistance. Furthermore, the differential expression of genes located within the previously described QTLs led to the identification of the gene MELO3C021395, which encodes the 33A subunit of the RNA Polymerase II transcription mediator (MED33A), as the candidate gene responsible for resistance against the virus.

These findings have enabled the development of closely linked molecular markers for WMV, CYSDV, and *Px* resistance. These markers have been used to create a breeding program to introgress all three resistances into commercial genetic backgrounds. Through backcrossing programs, marker-assisted selection, and metabolomic analyses, pre-breeding lines of yellow melons resistant to all three pathogens have been obtained, while still closely resembling the recurrent parental line. Currently, similar progress is being made in a breeding program aimed to improve the `Piel de sapo' type melon.

Resumen

Las cucurbitáceas son la segunda familia de hortícolas más importante a nivel mundial, solo por detrás de las solanáceas. Tradicionalmente su cultivo se ha llevado a cabo en las zonas templadas del planeta. Sin embargo, las condiciones de cambio climático, el comercio internacional y los modelos de agricultura intensiva favorecen la aparición de nuevas virosis y enfermedades fúngicas en zonas donde antes no estaban presentes. En este sentido, resulta esencial el monitoreo periódico de las principales zonas productoras, para así poder detectar los virus y hongos emergentes en cada territorio y adaptar los programas de mejora a los objetivos específicos de cada zona. En el caso concreto del melón (*Cucumis melo*) existe una gran variabilidad intraespecífica que puede servir como fuente de alelos de resistencia frente a estos patógenos. Sin embargo, las fuentes de resistencia suelen encontrarse dentro del germoplasma silvestre, normalmente originario de África o Asia, y en el que el nivel de domesticación es reducido. Para un mejor aprovechamiento de las accesiones resistentes, resulta necesario un estudio del control genético de los caracteres de interés, que permita localizar las regiones asociadas a la resistencia y diseñar marcadores moleculares asociadas a las mismas. Esto facilita los programas de mejora orientados a la introgresión de las resistencias manteniendo el fondo genético de las variedades de interés

En la presente tesis doctoral, durante las campañas de verano de 2019 y 2020, se ha llevado a cabo un estudio de la incidencia y diversidad genética de 9 especies virales potencialmente limitantes para el cultivo de cucurbitáceas en el sur este español. Se ha podido observar que los virus transmitidos por pulgones son prevalentes frente a los transmitidos por mosca blanca. Dentro del primer grupo destacó la presencia de watermelon mosaic virus (WMV), cucurbits aphid borne yellows virus (CABYV) y cucumber mosaic virus (CMV), ya que fueron detectados en todas las zonas y cultivos estudiados, apareciendo frecuentemente en infecciones mixtas. Moroccan watermelon mosaic virus (MWMV) y tomato leaf curl New Delhi virus (ToLCNDV) también fueron detectados en algunas zonas, pero con porcentajes de infección más bajos y normalmente en infecciones mixtas con WMV. Los análisis filogenéticos de los distintos aislados encontrados ha permitido la identificación de 7 nuevos perfiles moleculares de WMV y de aislados recombinantes de CMV, lo que es consistente con los resultados obtenidos en otros países y pone de manifiesto la gran variabilidad de estos patógenos.

Las accesiones silvestres de melón recogidas en distintos bancos de germoplasma son un valioso recurso para los programas de mejora genética frente a estreses bióticos. La accesión africana TGR-1551 ha sido descrita previamente como

resistente a WMV, CYSDV (cucurbit yellow stunting disorder virus), CABYV y el hongo *Podosphaera xanthii* (*Px,* razas 1, 2 y 5) agente causal del oídio en melón. Además, es tolerante a la mosca blanca (*Bemisia tabaci*) y portadora del gen *Vat* (*virus aphid transmission*), el cual limita la transmisión de virus por pulgón. Por lo tanto, esta accesión constituye una buena fuente de alelos de resistencia y, al poder utilizar un único parental donante, su uso acortaría los programas de mejora.

En el marco de la presente tesis doctoral, mediante el desarrollo de poblaciones segregantes de mapeo y el aprovechamiento de las tecnologías de genotipado masivo se han podido cartografiar los QTLs asociados a la resistencia a CYSDV derivados de esta entrada. En el caso de la resistencia a CYSDV, se han detectado dos QTL en el cromosoma 5. El primero de ellos es de efecto mayor y herencia dominante, estando asociado al desarrollo de síntomas. El segundo QTL, de efecto menor y también de herencia dominante, no confiere resistencia por sí mismo y está asociado a la carga viral durante la infección. Siguiendo una estrategia similar se han podido cartografiar y estrechar los QTLs de resistencia frente a *Px*. En este caso se trata de una epistasia dominante-recesiva, en la que el gen recesivo está localizado en el cromosoma 12 y el dominante en el cromosoma 5, concretamente en la misma región en la que se localiza el QTL mayor de resistencia a CYSDV.

Con relación a la resistencia frente a WMV, estudios previos realizados por el grupo de investigación mostraban que la misma estaba controlada por un gen recesivo en el cromosoma 11 y un QTL menor con efectos modificadores en el cromosoma 5. Con el fin de profundizar en el conocimiento de los mecanismos moleculares que dan lugar a la resistencia se ha llevado a cabo un análisis transcriptómico mediante RNA-seq. En este ensayo se ha comparado la respuesta diferencial de una línea con resistencia derivada de TGR-1551 y una susceptible frente a la infección con WMV. Los resultados mostraron un alto grado de remodelación transcriptómica en la línea resistente, activándose distintos mecanismos asociados con las rutas de defensa frente a patógenos, lo cual contrasta con el carácter recesivo de la resistencia. Por otra parte, la expresión diferencial de los genes localizados dentro de los QTLs previamente descritos ha permitido identificar el gen MELO3C021395, el cual codifica la subunidad 33A del mediador de la transcripción de la RNA polimerasa II (MED33A), como el gen candidato responsable de la resistencia frente al virus.

Estos resultados han permitido el desarrollo de marcadores moleculares estrechamente ligados a la resistencia frente a WMV, CYSDV y *Px*, los cuales han sido utilizados para desarrollar un programa de mejora genética para la introgresión de las tres resistencias en fondos genéticos comerciales. Mediante un programa de retrocruces, la selección asistida por marcadores y los análisis

metabolómicos, se han obtenido líneas de pre-mejora de melón amarillo resistentes a los tres patógenos, pero altamente similares al parental recurrente. Actualmente se continúa avanzando en un programa similar para la mejora del melón tipo piel de sapo.

Resum

Les cucurbitàcies són la segona família d'hortícoles més important a nivell mundial, només per darrere de les solanàcies. Tradicionalment el seu cultiu s'ha dut a terme a les zones temperades del planeta. No obstant això, les condicions de canvi climàtic, el comerç internacional i els models d'agricultura intensiva afavoreixen l'aparició de noves virosis i malalties fúngiques en zones on abans no estaven presents. En aquest sentit, resulta essencial el monitoratge periòdic de les principals zones productores, per a d'aquesta manera, poder detectar els virus i fongs emergents en cada territori i adaptar els programes de millora als objectius específics de cada zona. En el cas concret del meló (*Cucumis melo*) existeix una gran variabilitat intraespecífica que pot servir com a font d'al·lels de resistència enfront d'aquests patògens. No obstant això, les fonts de resistència solen trobar-se dins del germoplasma silvestre, normalment originari d'Àfrica o Àsia, i en el qual el nivell de domesticació és reduït. Per a un millor aprofitament de les accessions resistents, resulta necessari un estudi del control genètic dels caràcters d'interés, que permeta localitzar les regions associades a la resistència i dissenyar marcadors moleculars associats a aquestes. Això facilita els programes de millora orientats a la introgressió de les resistències mantenint el fons genètic de les varietats d'interés.

En la present tesi doctoral, durant les campanyes d'estiu de 2019 i 2020, s'ha dut a terme un estudi de la incidència i diversitat genètica de nou espècies virals potencialment limitants per al cultiu de cucurbitàcies en el sud-est espanyol. S'ha pogut observar que els virus transmesos per pugons són prevalents enfront dels transmesos per mosca blanca. Dins del primer grup va destacar la presència de watermelon mosaic virus (WMV), cucurbits aphid born yellows virus (CABYV) i cucumber mosaic virus (CMV), ja que van ser detectats en totes les zones i cultius estudiats, apareixent sovint en infeccions mixtes. Moroccan watermelon mosaic virus (MWMV) i tomatoleaf curl New Delhi virus (ToLCNDV) també van ser detectats en algunes zones, però amb percentatges d'infecció més baixos i normalment en infeccions mixtes amb WMV. Les anàlisis filogenètiques dels diferents aïllats trobats ha permés la identificació de set nous perfils moleculars de WMV i d'aïllats recombinants de CMV, la qual cosa és consistent amb els resultats obtinguts en altres països i posa de manifest la gran variabilitat d'aquests patògens.

Les accessions silvestres de meló recollides en diferents bancs de germoplasma són un valuós recurs per als programes de millora genètica enfront d'estressos biòtics. L'accessió africana *TGR-1551 ha sigut descrita prèviament com a resistent a WMV, CYSDV (cucurbit yellow stunting disorder virus), CABYV i el fong *Podosphaera*

xanthii (Px, races 1, 2 i 5) agent causal de l'oïdi en meló. A més, és tolerant a la mosca blanca (*Bemisia tabaci*) i portadora del gen Vat (virus aphid transmission), el qual limita la transmissió de virus per pugó. Per tant, aquesta accessió constitueix una bona font d'al·lels de resistència i, en poder utilitzar un únic parental donant, el seu ús acurtaria els programes de millora.

En el marc de la present tesi doctoral, mitjançant el desenvolupament de poblacions segregants de mapatge i l'aprofitament de les tecnologies de genotipat massiu s'ha pogut cartografiar els QTLs associats a la resistència a CYSDV derivats d'aquesta entrada. En el cas de la resistència a CYSDV, s'han detectat dues QTL en el cromosoma cinc. El primer d'ells és d'efecte major i herència dominant, estant associat al desenvolupament de símptomes. El segon QTL, d'efecte menor i també d'herència dominant, no confereix resistència per si mateix i està associat a la càrrega viral durant la infecció. Seguint una estratègia similar s'han pogut cartografiar i estrényer els *QTLs de resistència enfront de *Px*. En aquest cas es tracta d'una epistàsia dominant-recessiva, en la qual el gen recessiu està localitzat en el cromosoma dotze i el dominant en el cromosoma cinc, concretament en la mateixa regió en la qual es localitza el QTL major de resistència a CYSDV.

Amb relació a la resistència enfront de WMV, estudis previs realitzats pel grup d'investigació mostraven que la mateixa estava controlada per un gen recessiu en el cromosoma onze i un QTL menor amb efectes modificadors en el cromosoma cinc. Amb la finalitat d'aprofundir en el coneixement dels mecanismes moleculars que donen lloc a la resistència s'ha dut a terme una anàlisi transcriptòmica mitjançant RNA-seq. En aquest assaig s'ha comparat la resposta diferencial d'una línia amb resistència derivada de TGR-1551 i una susceptible enfront de la infecció amb WMV. Els resultats van mostrar un alt grau de remodelació transcriptòmica en la línia resistent, activant-se diferents mecanismes associats amb les rutes de defensa enfront de patògens, la qual cosa contrasta amb el caràcter recessiu de la resistència. D'altra banda, l'expressió diferencial dels gens localitzats dins dels QTLs prèviament descrits ha permés identificar el gen MELO3C021395, el qual codifica la subunitat 33A del mediador de la transcripció de la RNA polimerasa II (MED33A), com el gen candidat responsable de la resistència enfront del virus.

Aquests resultats han permés el desenvolupament de marcadors moleculars estretament lligats a la resistència enfront de WMV, CYSDV i *Px*, els quals han sigut utilitzats per a desenvolupar un programa de millora genètica per a la introgressió de les tres resistències en fons genètics comercials. Mitjançant un programa de retrocreuaments, la selecció assistida per marcadors i les anàlisis metabolòmiques, s'han obtingut línies de premillora de meló groc resistents als tres patògens, però

altament similars al parental recurrent. Actualment es continua avançant en un programa similar per a la millora del meló tipus Piel de Sapo.

General introduction

1. Cucurbitaceae family

1.1. Economic importance

The cucurbits family (*Cucurbitaceae*) is composed by 120 genera and more than 800 species, which are mainly cultivated in tropical and subtropical regions. The species found within this family are of great economic importance, only surpassed by some species of the *Solanaceae* family (Garcia-Mas & Puigdomènech, 2016). Among the most important crops within the *Cucurbitaceae* family we can find melon (*Cucumis melo* L.), watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai), cucumber (*Cucumis sativus* L.) and zucchini and pumpkins (*Cucurbita* spp.), whose annual worldwide production during 2021 was over 28, 101, 93 and 23 million tones, respectively (FAO, 2023). In all cases, except for *Cucurbita* spp., China was the main producer, but it could be observed that the countries located in the Mediterranean basin were in a priority position in the production of these fruits (**Figure 1**). Within the European Union (EU), Spain was the main producer of melon (652.600 t), watermelon (1.382.280 t), squashes and zucchini (789.780 t), and cucumber (745.910 t).

Within the Spanish territory, Andalucía is the main producer of watermelon, *Cucurbita* spp. and cucumber. Attending melon production, it is almost equally distributed among Andalucía, Región de Murcia and Castilla la Mancha. Comunidad Valenciana also plays an important role in cucurbits production, as it is the second producer of *Cucurbita* spp. and the fourth of melon and watermelon. Spanish´ cucurbits are mainly produced under irrigated conditions. Melon, watermelon, pumpkin and squashes were mostly produced in open field, whereas zucchini (*Cucurbita pepo* L.) and cucumber were mainly grown under green-house conditions in Almería (Andalucía). Spanish production is mainly assigned for export to other EU countries. For example, on average, Spain exports more than 430.000 t of melon (66.15% of its production) each year, which results in revenues of more than 312 million € (EUROSTAT, 2023). Additionally, the production of these crops has a high social incidence due to its capacity to generate jobs, representing a high concentration of workforce in the agricultural system. This is especially important in rural areas at risk of depopulation.

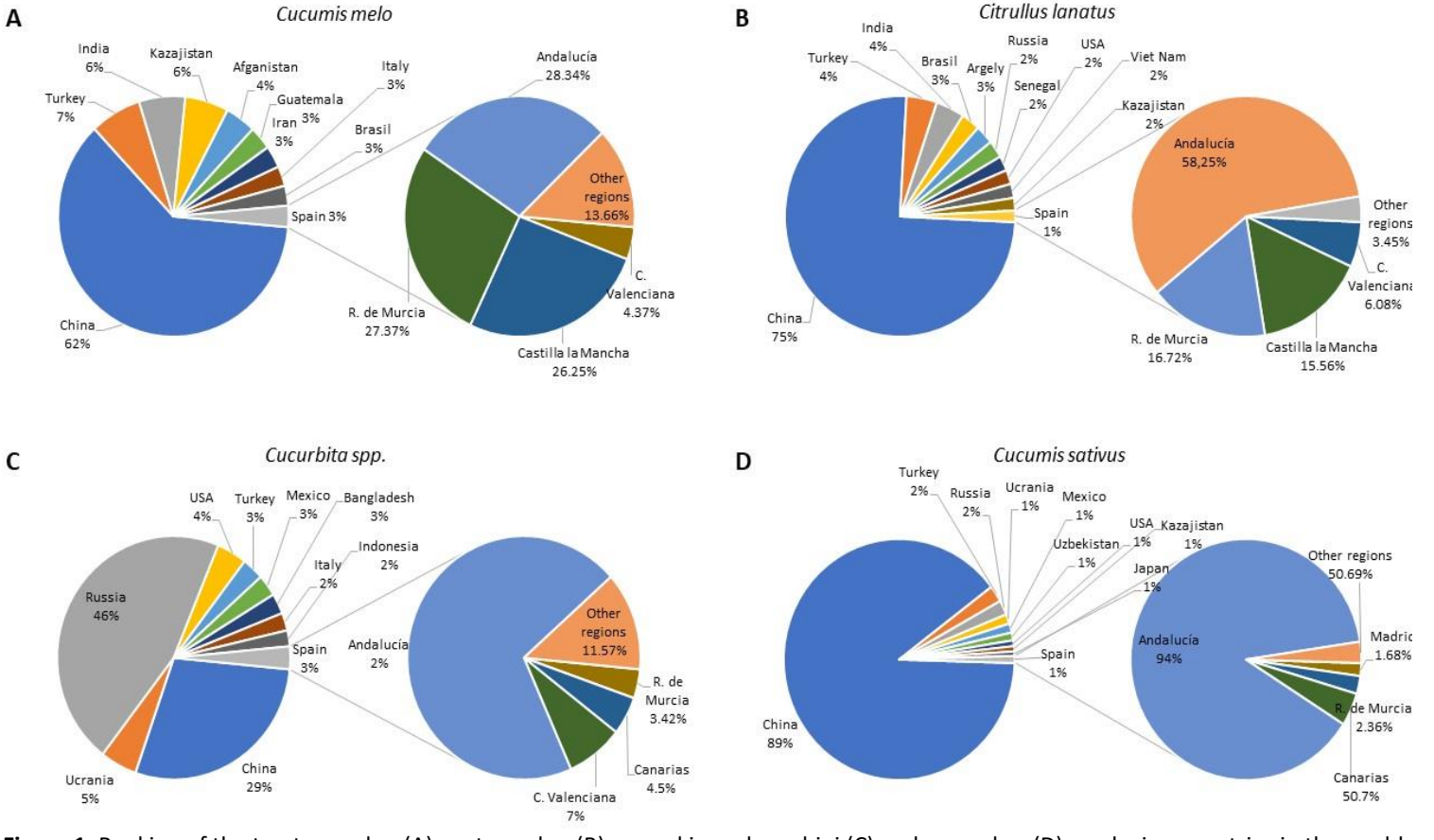

Figure 1. Ranking of the top ten melon (A), watermelon (B), pumpkin and zucchini (C) and cucumber (D) producing countries in the world. The percentages indicate the proportion produced by each country with respect to total world production. The top 5 producing regions within Spain are also indicated. Figure created from FAO (2023) data.

1.2. Main crop limitations

Cucurbits production has experienced an increase in yield and fruit quality over the last decades. This better performance of the crops can be explained by different factors. For example, an improvement in cultural practices has been observed thanks to the development of better irrigation systems, the digitalization of agricultural holdings or the use of green-houses, nitrogen fertilizers and hybrid seeds (Jones & Naidu, 2019; Pitrat, 2012). Nevertheless, its production is threatened by both abiotic and biotic factors, the incidence of which has increased due to climate change and globalization (Ghini *et al*., 2012; Pandey & Basandrai, 2021; Velasco *et al*., 2020). The increase in temperatures has highlighted the need to cultivate varieties better adapted to drier environments, where irrigation will be scarce (Elsayed *et al.*, 2019; Lesk *et al.*, 2016;). Additionally, the registered higher temperatures have led to outbreaks of pathogens in temper areas, where previously they were not a problem (Castro *et al.*, 2020; de Sousa Linhares *et al.*, 2020; Pandey & Basandrai, 2021; Pivonia *et al.*, 2002). Moreover, globalization and international commerce can favor the spread of these pathogens (Constable *et al.*, 2021).

Thereby, crops production, as well as plant breeding programs, should be adapted to these new conditions, with the final aim to produce enough food to feed an exponentially increasing population. Hereinafter, the principal biotic factors affecting cucurbits production are listed (**Figure 2**).

1.2.1. Pests

Among the principal pests causing losses in cucurbits crops, red spiders (*Tetranychus urticae* Koch and *Oligonychus mexicanus* McGregor & Ortega), aphids (*Aphis gossypii* Glover and *Myzus persicae* Sulzer), white-flies (*Bemisia tabaci* Gennadius and *Trialeurodes vaporarium* Westwood), nematodes (*Meloidogyne* spp., *Xiphinema americanum* Cobb and *Xiphinema rivesi* Damalso) and trips (*Frankliniella occidentalis* Pergande and *Thrips palmi* Karny) should be highlighted. These pathogens can reduce yields by feeding from the plant and, additionally, they can act as viral vectors (Messelink *et al.*, 2020).

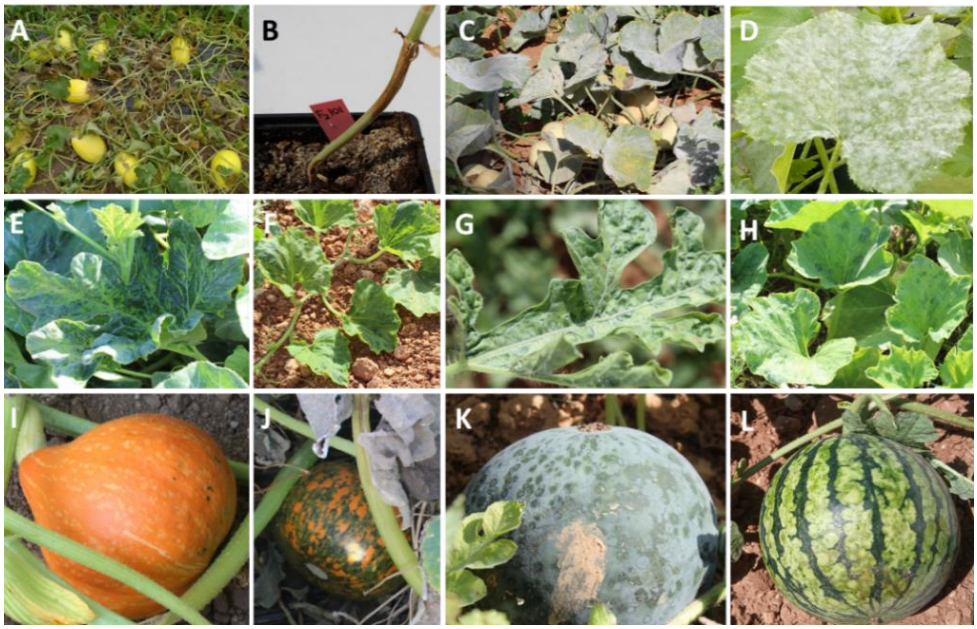

Figure 2. Symptoms produced by *M. cannonballus* (A), *M. phaseolina* (B), powdery mildew (C, D) and mixed viral infections (E-H) in cucurbits plants and fruits (I-L).

1.2.2. Viral infections

Viruses can be highlighted among the most important pathogens affecting agriculture. More than 59 viral species affecting cucurbits have been described worldwide (Velasco *et al.*, 2020), and 28 of them have already been identified within the Mediterranean basin (Lecoq & Desbiez, 2012; Radouane *et al.*, 2021). Lecoq & Katis (2014) estimated that approximately 1% of the worldwide cultivated cucurbits would be suffering a viral infection. Globally, aphid-transmitted viruses, including potyviruses such as watermelon mosaic virus (WMV), zucchini yellow mosaic virus (ZYMV), papaya ringspot virus (PRSV), and Moroccan watermelon mosaic virus (MWMV); cucumoviruses like cucumber mosaic virus (CMV); and poleroviruses such as cucurbit aphid-borne yellows virus (CABYV), are widely distributed and cause severe epidemics in major production areas (Bertin *et al*., 2020; Desbiez *et al*., 2020; Khanal *et al*., 2021; Nematollahi *et al*., 2021; Pozzi *et al*., 2020; Rabadán *et al*., 2021; Wang *et al*., 2017; Wang *et al*., 2019).

Whitefly-transmitted criniviruses, such as beet pseudo-yellows virus (BPYV), transmitted by *Trialeurodes vaporariorum*, were previously more significant until they were displaced by *Bemisia tabaci*. Currently, *B. tabaci*-transmitted criniviruses like cucurbit yellow stunting disorder virus (CYSDV), which caused severe economic losses in the late 1990s (Berdiales *et al.*, 1999; Lecoq & Desbiez, 2012; Tamang *et*

al., 2021), and the emerging cucurbit chlorotic yellows virus (CCYV), that was first reported in Europe in 2011 and that has rapidly spread (Kheireddine & Pico; Orfanidou *et al.*, 2014; Maachi *et al.*, 2022), as well as ipomoviruses like cucumber vein yellowing virus (CVYV) (Velasco *et al*., 2016), are causing more damage. Among the *B. tabaci*-transmitted viruses, begomoviruses have become a major problem in this crop family in the last decade, infecting cucurbits with varying severity. Tomato leaf curl New Delhi virus (ToLCNDV), which was reported in Europe for the first time in 2012 (Juárez *et al.*, 2014), is currently the most destructive representative of this group, although squash leaf curl virus (SqLCV), watermelon chlorotic stunt virus (WmCSV), and melon chlorotic mosaic virus (MeCMV) also pose significant threats (Martín-Hernández & Picó, 2020). The emergence of new races of the carmovirus melon necrotic spot virus (MNSV) (Gómez-Aix *et al.*, 2016), transmitted by the fungus *Olpidium*, is also increasing its incidence in association with this virus. Other mechanically transmitted viruses, such as tobamoviruses including cucumber green mottle mosaic virus (CGMMV) and zucchini green mottle mosaic virus (ZGMMV), are prevalent in specific areas (Crespo *et al.*, 2017). There are numerous other viruses confined to specific regions with negligible economic impact.

Although most studies on cucurbit viruses primarily focus on single infections, evidence shows that mixed infections are more common in nature (Moreno & López-Moya, 2020; Syller, 2012). The co-infection interaction of different viruses can exacerbate symptom development and lead to increased viral accumulation in the plant (Syller, 2012; Tatineni *et al.*, 2020). In cucurbits, the synergistic effect of mixed infections between viruses of different genera has been studied. The interaction between CMV and two potyviruses (ZYMV and WMV) has been characterized in melon, zucchini, *C. maxima* Duchesne, and watermelon, revealing changes in the concentration levels of these viruses in the plant (Wang *et al*., 2019; Wang *et al*., 2002). Abrahamian *et al*. (2015) observed an increase in symptom severity in cucumber due to mixed infections of criniviruses and begomoviruses. Recently, Domingo-Calap *et al*. (2020) described how the concurrent infection of CYSDV and WMV in melon benefits both viruses, facilitating and maintaining their transmission. Moreover, mixed infections also promote the emergence of new recombinant isolates (Syller, 2012). These recombination events and exchange of genetic material have been identified as the main triggering factors for variability in viruses, enabling evolution and adaptation to new hosts and the formation of new virus species (Xiao *et al.*, 2016; Moriones *et al.*, 2017).

Here below, the principal characteristics of WMV and CYSDV, two important virus infecting cucurbits, are described.

1.2.2.1. Watermelon mosaic virus.

Watermelon mosaic virus (WMV, genus *Potyvirus*, family *Potyviridae*) is a globally distributed potyvirus known for its extensive host range (**Figure 3A**).It is transmitted by aphids in a non-persistent manner and infects over 170 plant species belonging to 26 families, including cucurbits and legumes (Sharifi *et al.*, 2008). WMV infection leads to the development of characteristic symptoms such as mosaic patterns, leaf deformations, and discoloration of both leaves and fruits (**Figure 3B**). These symptoms have severe implications for infected crops, resulting in significant reductions in both yield and quality.

WMV has a flexuous filamentous virion structure, consisting of a single-stranded positive-sense RNA genome, meaning that the RNA can serve as a template for protein synthesis directly. The genome of WMV is approximately 10,000 nucleotides in length and contains all the necessary genetic information for viral replication, movement within the host plant, and the induction of disease symptoms (Desbiez & Lecoq, 2004) (**Figure 3C**). Genomic RNA undergoes translation to yield two polyproteins that require proteolytic processing to generate ten mature proteins and one fusion protein (Merits *et al.*, 2002). These proteins are vital for replication and movement of the virus. The translated polyproteins include P1, which serves as a translation modulator and also plays a role in replication (Verchot & Carrington, 1995). Another protein, helper component proteinase HC-Pro, acts as a silencing suppressor and facilitates aphid transmission (Govier *et al.*, 1977; González-Jara *et al.*, 2005; Soitamo *et al.*, 2011). P3 is involved in both virus replication and movement (Cui *et al*., 2009; Klein *et al*., 1994), while P3N-PIPO enables cell-to-cell movement (Wen & Hajimorad, 2010; Vijayapalani *et al.*, 2012). The proteins 6K1 and 6K2 contribute to the formation of replication vesicles (Cui & Wang, 2016; Geng *et al.*, 2017). Cytoplasmic inclusion protein (CI) functions as a helicase involved in virus movement and replication (Fernández *et al.*, 1997; Gabrenaite-Verkhovskaya *et al.*, 2008). The genome-linked protein VPg is involved in translation, virus movement, and replication (Rantalainen *et al.*, 2011). NIa-Pro is responsible for polyprotein processing (Dougherty & Parks, 1991), NIb serves as an RNA-dependent RNA polymerase (Hong & Hunt, 1996), and CP functions in virus movement, virion formation, and aphid transmission (Revers & García, 2015).

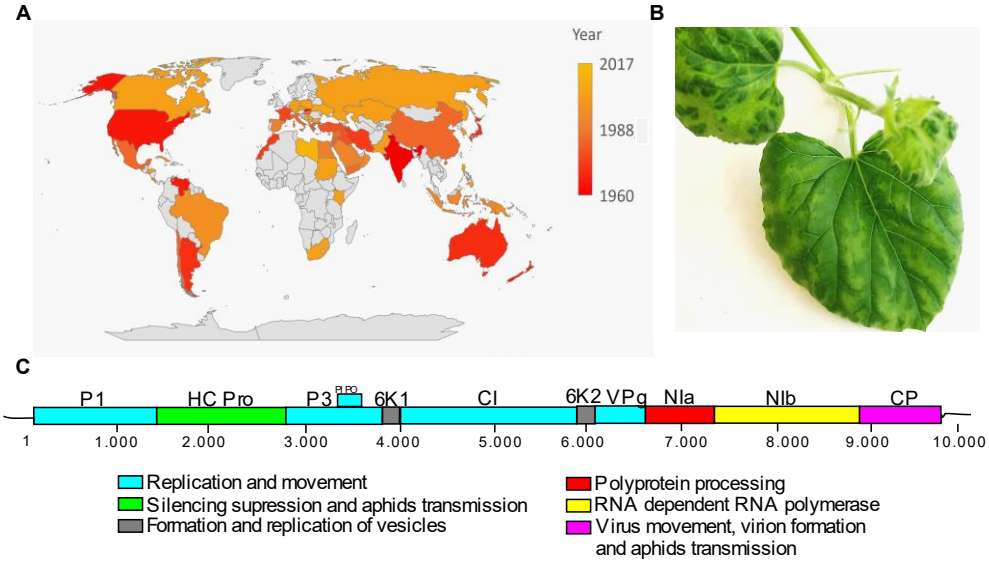

Figure 3. Global distribution of watermelon mosaic virus (WMV) indicating the year when it was first reported infecting cucurbits (A). Symptoms caused by WMV in melon (B). Genomic structure of WMV RNA. The different proteins conforming its polyprotein alongside with their main function is indicated (C).

Genome organization and protein functions in potyviruses exhibit a high degree of conservation (Gibbs & Ohshima, 2010; Revers & García, 2015). Nevertheless, certain variable regions have been identified in specific species (Johansen *et al.*, 1996; Adams *et al.*, 2005). For instance, in plum pox virus (PPV), the N-terminal region of P1 demonstrates hypervariability and plays a critical role in virus replication, host defense responses, and pathogenicity, exhibiting host-dependent effects (Maliogka *et al.*, 2012; Pasin *et al.*, 2014). In other potyviruses, genetic variations contribute to host adaptation, host-dependent pathogenicity, vector transmissibility, and viral accumulation in different hosts (Johansen *et al.*, 1996; Tan *et al.*, 2005; Moury *et al.*, 2011).

WMV exhibits significant genetic diversity. Ever since the year 2000, the `emergent' groups (EM1-4), that appeared for the first time in France, have completely displaced the `classic´ isolates that used to be predominant in the Mediterranean area (Bertin *et al*., 2020; Desbiez *et al*., 2009, 2020). These 4 EM groups appeared 20 years ago mainly due to recombination events and were associated to more severe symptoms. Recently, Desbiez *et al*. (2020) described new recombinant strains, some of which seem to overcome the previously described resistances (Desbiez *et al*., 2021). Understanding the genetic diversity of WMV is crucial for developing effective management strategies and breeding resistant cultivars.

However, even though WMV is a prevalent virus in the main Spanish producing areas (Juarez *et al.*, 2013; De Moya-Ruiz *et al.*, 2021), no wide phylogenetic studies have recently been conducted in to study its genetic variability in this region.

1.2.2.2. Cucurbit yellow stunting disorder virus.

Cucurbit yellow stunting disorder virus (CYSDV; genus *Crinivirus*, family *Closteroviridae*) is a common virus infecting cucurbits fields in many tropical and subtropical regions, including North and Central America, the Middle East and Mediterranean basin (Abou-Jawdah *et al.*, 2000; Brown *et al.*, 2007; Célix *et al.*, 1996; Kao *et al.*, 2000; Lecoq & Desbiez, 2012; Louro *et al.*, 2000; Tzanetakis *et al.*, 2013; Wisler *et al.*, 1998;) (**Figure 4A**). It is exclusively transmitted in a semipersistent manner by *B. tabaci* (Célix *et al.*, 1996) and, in addition to cucurbits, it can also infect alfalfa (*Medicago sativa* L.), lettuce (*Latuca sativa* L.) and snap bean (*Phaseolus vulgaris* L.). In cucurbits, symptoms associated to CYSDV are primarily characterized by interveinal chlorosis or yellowing, while major veins tend to retain green color until later stages of disease progression (**Figure 4B**) (Célix *et al.*, 1996; Tzanetakis *et al.*, 2013). Initial symptoms may also manifest as a yellow-green chlorotic mottle. These symptoms typically emerge on older leaves and gradually spread acropetally over time. In the case of melon or watermelon plants, symptom development commences with leaves near the crown and gradually extends along the vines. Although fruits do not exhibit obvious symptoms, studies have shown reduced sugar levels (ºBrix) in CYSDV-infected plants (Abou-Jawdah *et al.*, 2000; López-Sesé & Gómez-Guillamón, 2000). These symptoms resemble those induced by other yellowing viruses, including various members of the *Crinivirus* genus, and can also be mistaken for the symptoms caused by a lack of nutrients. CYSDV infections may cause a 80% yield reduction in melon (Abou-Jawdah *et al.*, 2000; Brown *et al.*, 2007; López-Sesé & Gómez-Guillamón, 2000).

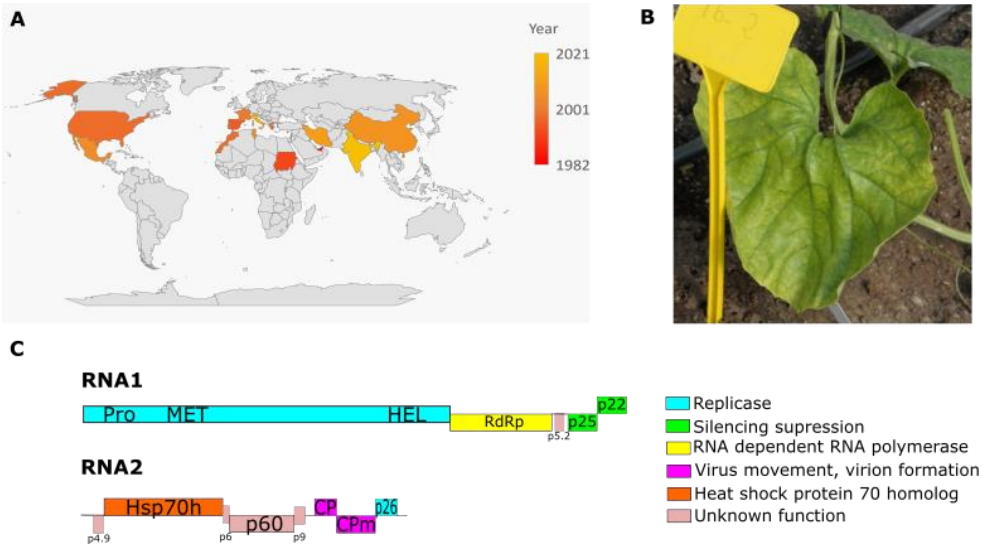

Figure 4. Global distribution of cucurbit yellow stunting disorder virus (CYSDV), indicating the year when it was first reported in cucurbits in each country (A). Symptoms caused by CYSDV in melon (B). Genomic structure of CYSDV, representing the proteins that conform each RNA and their main functions (C).

As other cucurbits criniviruses, the virion particles of CYSDV exhibit a flexuous, filamentous morphology with cross-banding and an open helix pattern. The genome of CYSDV is segmented in two positive-sense single-stranded RNA molecules (RNA1 and RNA2), each of which is essential for infectivity and is encapsidated separately (**Figure 4C**) (Célix *et al.*, 1996; Aguilar *et al.*, 2003; Navas-Castillo *et al.*, 2014). The first open reading frame (ORF) in RNA1, known as ORF1a, is responsible for encoding the RNA replicase. This polyprotein comprises the protease, methyltransferase, and helicase domains (Aguilar *et al.*, 2003). ORF1b encodes a protein that contains conserved motifs found in RNA-dependent RNA polymerases (RdRp) of positive-strand RNA viruses. In the case of RNA2, it encompasses ORFs that make up the hallmark closterovirus gene array, also known as the closterovirus quintuple gene block (Livieratos & Coutts, 2002; Aguilar *et al.*, 2003). This gene array includes a small hydrophobic protein of approximately 5-kDa, a heat shock protein 70 family homolog (Hsp70h), a protein of approximately 60-kDa, the major coat protein (CP), and a diverged or minor CP (CPm). The CP and CPm play crucial roles in virion formation and are the most abundant protein components (Agranovsky *et al.*, 1995). Both RNA1 and RNA2 contain several other ORFs, some of which encode putative small proteins, but their functions remain largely unknown. However, it has been demonstrated that the p22 and p25 proteins are both gene silencing supressors (Kataya *et al.*, 2009), while p5 contains

a N-terminal signal peptide that likely targets the endoplasmatic reticulum (Aguilar *et al.*, 2003).

CYSDV exhibits remarkably low genetic diversity compared to other members of the *Closteroviridae* family. The genetic characterization of isolates collected from the Middle East and Mediterranean Europe, focusing on the HSP70h and CP genes, revealed two distinct subpopulations (Rubio *et al*., 1999, 2001). The Western group consisted of isolates from Spain, Jordan, Lebanon, North America, and Turkey, while the Eastern group predominantly comprised isolates from Saudi Arabia. Nucleotide identity within the same group exceeded 99%, whereas the identity between the two groups was approximately 92%. Notably, a CYSDV population sampled in the geographic region where the virus was initially detected in Spain exhibited exceptional uniformity over an eight-year period (Marco & Aranda, 2005). In fact, the nucleotide diversity at synonymous positions in the coding regions of the CYSDV genome was approximately 30 times smaller than a comparable estimate for a population of tobacco mild green mosaic virus, a tobamovirus that is typically considered as very stable (Fraile *et al*., 1996; Marco & Aranda, 2005).

1.2.3. Fungal infections

In general, fungal infections are the principal biotic factor limiting crops productions, followed by viral infections (Loebenstein & Katis, 2014). Among the fungi that affect the root system of cucurbits, the most important are *Monosporascus cannonballus* Pollack & Uecker (Diatrypaceae) and *M. eutypoides* (Petrak), which are the causal agents of collapse; *Macrophomina phaseolina* (Tassi) Goid (Botryosphærieceae), the causal agent of carbonaceous rot; and vascular fusariosis (Fusarium wilt) caused by *Fusarium oxysporum* f. sp. *melonis* (Leach & Currence) W.C. Snyder & H.N. Hansen, *F. oxysporum* f.sp. *niveum* (E.F. Smith) W.C. Snyder & H.N. Hansen*, F. oxysporum* f.sp. *cucumerinum* J.H. Owen and *Neocosmospora* (*Fusarium) solani* (Mart.) Sacc. f.sp. *cucurbitae* W.C. Snyder and H.N. Hansen (González *et al.*, 2020). Some of these pathogens are thermophiles and the rising temperatures has led to an increased presence of these fungi in temper areas, where they had not been a problem to date (Castro *et al*., 2020; de Sousa Linhares *et al*., 2003; Pivonia *et al*., 2002).

Regarding fungal infections affecting the areal part of the plant (stem, leaves and fruits), it is worth naming *Phytophthora capsici* (Leonian), that causes fruit rot, downy mildew caused by *Pseudoperonospora cubensis* (Berk. &Curt.), and cucurbits powdery mildew (CPM), mainly produced by *Podosphaera xanthii* (Castagne) U. Braun & N. Shish. and *Golovinomyces orontii* (Castagne).

1.2.3.1. Cucurbits powdery mildew

As previously stated, *P. xanthii* (*Px*) and *G. orontii* (*Go*) are the principal causal agents of CPM. This is one of the most important fungal diseases, since the occurrence of these pathogens decreases both yield and fruit quality due to a reduction in the photosynthetic capacity (Cohen *et al*., 2004; Lebeda *et al*., 2007, 2009). The appearance of *Px* is favored by relatively high temperatures and humidity, whereas *Go* requires lower temperatures (Pirondi *et al.*, 2015). Thereby, CPM has a global distribution and affects both field and green-house conditions, with *Px* been predominant in the main producing areas worldwide. Moreover, the causal agents of CPM have a great capacity to mutate to more virulent races and/or pathotypes that can overcome the previously described genetic resistances (McCreight, 2006). In melon, to date, the use of a set of differential melon lines (Lebeda *et al*., 2016) has led to the identification of more than 25 *Px* races (Cui *et al*., 2022)(**Table 1**). Among them, races 1, 2, 3.5, 4 and 5 are the most common and the ones that have been described in Spain so far (Del Pino *et al.*, 2002; Torés *et al.*, 2009). However, the number of physiological races and their relative spatial distribution are likely to be greater. In this regard, the absence of current studies on the variability of races in many of the main producing areas, such as China, Central and South America and Europe, is striking. Notably absent are also reports from India, which is a rich source of CPM-resistant germplasm (Dhillon *et al.*, 2012). Systematic prospections on CPM variability only have recently been conducted in South Korea and Czech Republic (Hong *et al*., 2018; Lebeda *et al*., 2009). Studies carried out in Czech Republic showed that their CPM populations were unique and highly variable, as 56 new races were identified there between 2000 and 2009. Thereby, it would be important to conduct similar prospections in other producing areas, to better understand the actual status of CPM infections and thus be able to design better control strategies against this fungus.

Table 1. *Podosphaera xanthii* races affecting melon worldwide.

Race	Countries where it has been detected	First reported	References
0	France, Italy	1997	(Bardin *et al.*, 1997; Miazzi *et al.*, 2011)
1	USA, Brazil, Spain, Israel, Greece, Japan, Tunisia, Sudan, Cyprus, South Korea, China	1926	(Cohen & Eyal, 1988; de Oliveira-Rabelo *et al.*, 2017; del Pino *et al.*, 2002; Hong *et al.*, 2018; Hosoya *et al.*, 2000; Jagger & Scott, 1937; McCreight, 2006; Mohamed *et al.*, 1995; Ning *et al.*, 2014; Vakalounakis & Klironomou, 1995)
2	USA, Brazil, Spain, Israel, Greece, Japan, Turkey, Senegal, Sudan, Italy	1938	(Cohen & Eyal, 1988; de Oliveira-Rabelo *et al.*, 2017; Del Pino *et al.*, 2002; Jagger, 1938; McCreight, 2006; Vakalounakis & Klironomou, 1995)
2F	France, China, Italy	1984	(McCreight *et al.*, 1987; Miazzi *et al.*, 2011; Zhou *et al.*, 2020)
2U.S.	USA	1984	(McCreight *et al.*, 1987; McCreight & Coffey, 2011)
3	USA, India, Israel, Brazil, Italy	1976	(Thomas, 1978; Kaur & Jhooty, 1985; Cohen *et al.*, 1996; Miazzi *et al.*, 2011; de Oliveira-Rabelo *et al.*, 2017)
3.5	USA, Spain	2005	(Torés *et al.*, 2009; McCreight & Coffey, 2011)
4	France, Czech Republic, Israel, Brazil, Spain, Italy	1986	(Bardin *et al.*, 1997; Del Pino *et al.*, 2002; Cohen *et al.*, 2004; Miazzi *et al.*, 2011; McCreight *et al.*, 2012; de Oliveira-Rabelo *et al.*, 2017)
4.5	France, USA	2008	(Pitrat & Besombes, 2008; McCreight & Coffey, 2011)
5	France, Czech Republic, Israel, USA, Spain, Japan, Italy	1996	(Bardin *et al.*, 1997; Hosoya *et al.*, 2000; Del Pino *et al.*, 2002; Cohen *et al.*, 2004; Miazzi *et al.*, 2011; McCreight *et al.*, 2012)
6	France	2002	(Bertrand, 2002)
P6	USA	2005	(McCreight *et al.*, 2012b)
N1	Japan, South Korea	2000	(Hosoya *et al.*, 2000; Hong *et al.*, 2018)
N2	Japan, South Korea, China	2000	(Hosoya *et al.*, 2000; Hong *et al.*, 2018)(Li *et al.*, 2019)
N3, N4	Japan	2000	(Hosoya *et al.*, 2000)
N5	Japan	2009	(Kim *et al.*, 2016b)
F	Czech Republic	2003	(Lebeda & Sedláková, 2006)
G	Czech Republic	2003	(Lebeda & Sedláková, 2006)
H	Czech Republic	2003	(Lebeda & Sedláková, 2006)
S	USA, Japan	2003	(Izumikawa *et al.*, 2008; McCreight & Coffey, 2011)
A	Japan, South Korea	2008	(Izumikawa *et al.*, 2008; Kim *et al.*, 2016)
O	Japan	2008	(Izumikawa *et al.*, 2008)
Ch1	China	2010	(Liu *et al.*, 2010a)
KN1, KN2	South Korea	2018	(Hong *et al.*, 2018)

Traditionally, the main control measure against CPM has been the use of fungicides, nevertheless, since 1967, resistance of *Px* to different systemic fungicides has frequently been reported (Schroeder, 1969). Nowadays, the most effective fungicides against CPM belong to the group of Sterol demethylation inhibitor fungicides, which requires polygenic changes of the pathogen to become resistant (Pérez-García *et al.*, 2009). However, resistant isolates of *Px* to these kind of fungicides have already been reported (Vielba-Fernández *et al.*, 2020). Recent environmental concerns, and the increasing production of organic crops, has led to the development of several biological control agents against CPM. These compounds, which encompassed *Bacillus* bacteria, epiphytic fungi, and other mycoparasitic microorganisms, exhibited a reduction in symptoms ranging from 25% to 75% in efficacy (Lee & Park, 2021; Li & Punja, 2020; Panstruga *et al*., 2015; Safaei *et al*., 2021). Nevertheless, these organic products have only been tested on cucumber grown under green-house conditions and further studies should be assessed to test if they are efficient and cost-effective in open-field conditions.

The current trend in pathogen management is based on an integrated control strategy, by combining the effects of chemical and biological control, good cultural practices and the use of resistant varieties (Cui *et al.*, 2022). Plant breeding is making great efforts towards producing varieties with durable resistances against CPM. However, the resistance against a single physiological race has a limited usefulness, since multiple races can be found in a same field and a single resistance can be easily overcome by the appearance of new races. Studies conducted in California (USA) showed a general race stability (McCreight *et al.*, 2012). Thereby, breeding efforts should be directed to the development of multi-resistant lines to control the predominant races in each region. Hence, each country would have different sets of resistance genes, as it does horticultural and market-specific traits (McCreight *et al.*, 2012). This goal can be achieved either by pyramiding resistance genes from different accessions or by the use of multi-resistant accessions as donor parents.

2. Breeding melons resistant to WMV, CYSDV and CPM

2.1. Melon genetic variability

According to recent studies, the species *C. melo* is believed to have emerged around 3 thousand years ago (Endl *et al.*, 2018; Sebastian *et al.*, 2010). However, the exact location of its domestication has been a subject of ongoing debate (Thakur *et al.*, 2019; Yashiro *et al.*, 2005). Initially, it was hypothesized that *C. melo* originated in Africa, followed by domestication processes in the Middle East and India (Robinson & Decker-Walters, 1997). However, since wild melon varieties have been observed

in both Asia and Africa, it is possible that two independent domestication processes occurred concurrently. Furthermore, Endl *et al*. (2018) and Sebastian *et al*. (2010) proposed the existence of an additional Australian group. Another theory suggests that there were three separate domestication events, one in Africa and two in India (Zhao *et al*., 2019). The domestication processes observed in India took place through different routes, but similar phenotypes were achieved. In any case, it seems clear that the domestication process that took place in Africa only resulted in the tibish cultivar (**Figure 5**), which is just consumed in the North of this continent (Gonzalo *et al*., 2019). On the other hand, the domestication event or events that happened in Asia resulted in a wide diversity of melon horticultural groups that rapidly spread throughout the Mediterranean basin (Gonzalo *et al*., 2019; Sabato *et al*., 2019).

Melon has undergone a great diversification process since its origin, which is mainly evidenced by the great variability observed in fruit morphology and physiology traits. India is considered as the primary diversification center for commercial melon cultivars, whereas Japan, Afghanistan, Iran, Iraq, Turkey, Portugal and Spain are secondary diversification centers (Gonzalo *et al*., 2019; Robinson & Decker-Walters, 1997). Melons were introduced into Europe by three routes: Eastern Europe, the Balkans and Italy. In the case of Spain, it is thought that not-sweet melons with an elongated phenotype (flexuosus group) appeared firstly. It was not until the Islamic Conquest that sweet melons were introduced in the Iberian Peninsula (Paris *et al*., 2012).

Based on differences in ovary hairs, melon (*C. melo*) has traditionally been classified within two subspecies: subsp. *melo* (with long hairs on the ovary) and subsp. *agrestis* (with short hairs on the ovary) (Jeffrey, 1980). Nevertheless, the species *C. melo* includes both wild-type and cultivated melons, which is translated into a great phenotypic variability, especially in traits related to fruit quality, shape, size, color, sweetness or aromas. Pitrat, (2016a), using descriptors previously employed, reclassified all the intraspecific variability in 19 groups (**Figure 5**): agrestis, kachri, chito, tibish, acidulus, momordica, conomon, makuwa, chinensis, flexuosus, chate, dudaim, chandalak, indicus, ameri, cassaba, ibericus, inodurus and cantalupensis. All this genetic variability is well preserved in different germplasm collections.

Considering the strong sexual compatibility barriers between *C. melo* and other related species, this great intraspecific variability is especially valuable (Pitrat, 2016a). In this regard, most of the resistances reported against fungal and viral pathogens have been described in a limited number of exotic accessions, which belong to agrestis, mormordica, kachri and acidulous groups, that are original from

India or Africa, as well as to conomon, makuwa and chinensis groups, that were originated in China, Japan and Korea (Pitrat, 2012, 2016a, 2016b). Especially interesting for breeding purposes are those accessions resistant to more than one viral or fungal pathogen, such as TGR-1551 (PI 482420), PI 414723, PI 161375 (Songwhan Charmi) or PI 313970. These genotypes have been widely studied and could be used to introgress their resistances into commercial varieties.

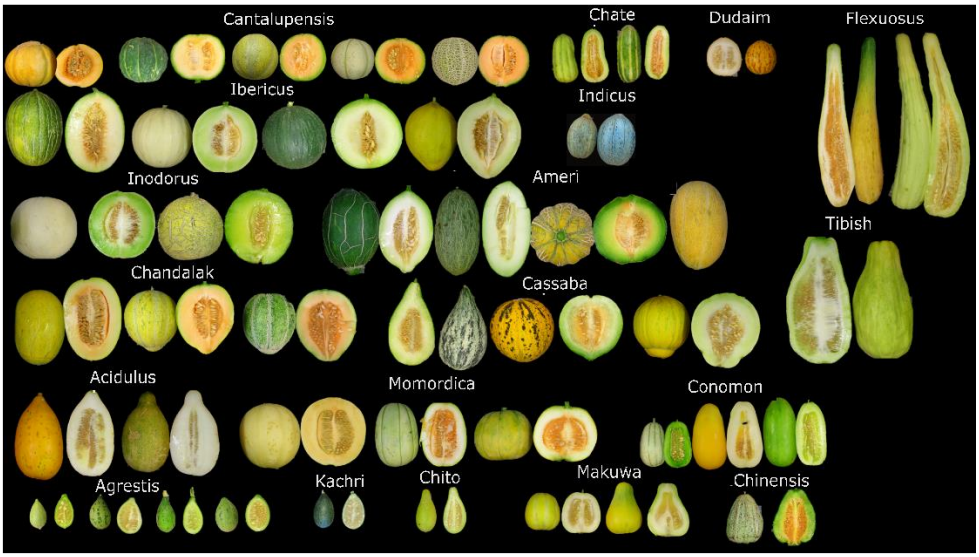

Figure 5. Main botanical groups into which the species Cucumis melo is divided, according to Pitrat (2016a). All the images have been kindly provided by Dr. Gorka Perpiñá.

2.1.1. Melon resistance sources against viruses

As previously stated, one of the most important objectives for breeders is to obtain melon varieties resistant to viruses. One of the first approaches to reach this goal was to study accessions resistant to viral vectors. A widely used strategy to breed commercial cultivars consists in the introgression of the *vat* gen (*Virus aphid transmission resistance gen),* which confers resistance to the transmission of viruses by *Aphis gossypii* (Glover) (*Homoptera, Aphididae*). This gen was originally discovered in the Korean accession PI 161375 (Lecoq *et al*., 1979) and, after that, hundreds of accessions were tested (Sarria-Villada *et al.*, 2009; Soria *et al.*, 2003; Fergany *et al.*, 2011; Pitrat *et al.*, 1996). These studies showed that about 5% of accessions were resistant to *A. gossypii* colonization. Among them were the accessions PI 414723 and TGR-1551. Further studies showed that this resistance was not only due to a single gen, but to a cluster of CC-NLR (coiled-coil containing nucleotide-binding leucine-rich repeat receptors) genes located on chromosome 5.

These R genes can recognize an effector present in the aphid's saliva and initiate an immune response characterized by enhanced peroxidase activity, as well as the deposition of callose and lignin in the cell walls neighboring the stylet pathway, thereby disrupting aphid behavior. However, the *vat* gene is not completely effective in preventing the transmission of viruses in the field. There may be other aphid species, such as *Myzus persicae* (Sulzer), that are not affected by this resistance and can also act as vectors (Castle *et al.*, 1992). Additionally, the resistance can be overcomed with massive infections of *A. gossypii* (Soria *et al.*, 2003).

Resistance against *B. tabaci* has also been observed in some accessions, such as TGR-1551, PI 414723, PI 164723 or PI 161375 (Boissot *et al*., 2003; Soria *et al*., 1999; Wintermantel *et al*., 2017). For these genotypes, the number of adults flies as well as the larval density was significative lower than in susceptible cultivars. It was suggested that the density and number of eggs would be negatively corelated to the number of trichomes (Baldin *et al.*, 2012). A RIL (recombinant inbred lines) population derived from PI 161375 was evaluated to map the resistance to *B. tabaci* (Boissot *et al*., 2010). Two additive QTLs (quantitative trait locus) were detected on chromosomes 7 and 9, but they only explained 17.9% and 13.8% of the variability, respectively, due to the low heritability of the character.

Considering the limited usefulness of resistances to these vectors, efforts should be focused on mapping and introgressing genetic resistances to the viruses themselves. In this sense, most of the resistances to virus diseases in melon have been described in a small number of exotic or wild accessions belonging to the agrestis, mormodica, kachri and acidulous groups from India or Africa, and to the conomon, makuwa and chinensis groups from Far-East (China, Japan and Korea) (Pitrat, 2012, 2016a, 2016b). Recently, Martín-Hernández & Picó (2020) published a review about the available melon resistance accessions against the most important viruses infecting cucurbits. Some of those genotypes, such as TGR-1551, PI 414723, PI 161375 or PI 313970, have been described as resistant to different viruses and have been used as resistant sources in breeding programs.

2.1.1.1. Melon resistance sources against WMV

Regarding WMV, several tolerant accessions have been described (Moyer *et al.*, 1985; Munger, 1991; Provvidenti *et al.*, 1978; Webb, 1967), but only two accessions, TGR-1551 and PI 414723, have been described as resistant to this potyvirus. In the momordica accession PI 414723 the resistance is controlled by a single dominant gene named *Wmr* (Gray *et al.*, 1986). The resistance offered by this gene is not complete, as plants show infection symptoms and they further recover

(Gilbert *et al.*, 1994), which is not useful in breeding programs. On the other hand, the African acidulous accession TGR-1551 remains asymptomatic during all the infection stages (Díaz-Pendón *et al*., 2003, 2005; Sarria-Villada *et al*., 2009; Soria *et al*., 2003), and it has proven to be resistant against both `classic' and `emerging´ WMV isolates (Pérez-de-Castro *et al.*, 2019). Resistance from TGR-1551 is controlled by one recessive gene together with other additional genetic factors (Díaz-Pendón *et al*., 2003, 2005). The phenotyping and genotyping analysis of an F_2 population resulting from the initial cross between TGR-1551 and the susceptible cultivar `Bola de oro´ (BO) enabled the mapping of a recessive major QTL on chromosome 11, along with the identification of several minor QTLs (Díaz-Pendón *et al.*, 2005). Subsequently, these findings were validated through the examination of a RILs population derived from the same initial cross. The candidate interval on chromosome 11 was then narrowed down to a region spanning 760 kb (Palomares-Rius *et al.*, 2011). Subsequent genotyping by sequencing of the RIL population, followed by the analysis of backcrosses derived from selected RILs, led to a further refinement of this QTL to a 140 kb interval. Moreover, the modifier effect of a minor QTL mapped on a 700 kb region on chromosome 5 was confirmed. For those plants that were heterozygous for the chromosome 11 allele but homozygous for the TGR-1551 allele on chromosome 5 a reduction in symptomatology was observed (Pérez-de-Castro *et al.*, 2019).

Gonzalez-Ibeas *et al*. (2012) conducted differential expression studies to investigate TGR-1551 response to WMV. This microarray assay revealed a greater number of transcriptomic modifications in resistant plants when compared to susceptible plants. These changes were associated with a defense response, which contrasts with the recessive nature of the resistance. Moreover, it has also been stated that silencing mechanisms could also be implied in the resistance response (Gonzalez-Ibeas *et al.*, 2011).

2.1.1.2. Melon resistance sources against CYSDV

Concerning CYSDV, several resistance sources have been identified: Ames 26704, PI 116482, PI 122847, PI 123496, PI 145594, PI 145596, PI 313970, TGR-1551, TGR-1937, PI 614185 and PI 614213 (López-Sesé & Gómez-Guillamón, 2000; McCreight *et al*., 2016; Wintermantel *et al*., 2017).

Based on controlled inoculations with a Spanish isolate, the resistance of TGR-1551 to CYSDV was described as controlled by a dominant gene, known as *cys*, with modulating factors (López-Sesé & Gómez-Guillamón, 2000). However, when inoculated with a Texas isolate, the resistance exhibited a recessive control (Gómez-Guillamón *et al.*, 2002). On the other hand, the resistance derived from PI

313970 was reported as recessive in naturally-infected field tests in California (McCreight & Wintermantel, 2011). Resistance in TGR-1551 was subsequently tested in the same field conditions and it was also found to be recessive (McCreight *et al.*, 2019). To combine their resistances, PI 313970 and TGR-1551 were crossed in an attempt to initiate a breeding program; however, no increased levels of resistance were observed in the field selections. Analyzing disease reaction data from the two resistance sources, as well as their F_1, F_2, and testcross progenies in naturally-infected field tests in California, suggested that the resistance to CYSDV exhibited by these two lines may be governed by allelic genes (McCreight *et al.*, 2019). The reasons for the differential pathogenicity of isolates in TGR-1551 remains unclear.

Regarding the TGR-1551 resistance to Spanish isolates, virus resistance was attributed to either the restriction of movement through the vascular system or the limitation of its accumulation levels (Marco *et al*., 2003). Recently, the genotyped by sequencing RIL population derived from TGR-1551 was used by Palomares-Ríus *et al*. (2016) to identified two QTLs linked to resistance against CYSDV on chromosome 5 (*cysdvq5.1* and *cysdvq5.2*). One of them appeared to be associated with symptom presence/absence, while the other was related to the virus content in the plant. Thus, there may be two genomic regions involved in the resistance, one responsible for restricting virus multiplication in the plant, and the other associated with virus movement and disease symptomatology, possibly linked to the virus's ability to disperse within the plant and induce yellowing symptoms.

2.1.2. *Melon resistance sources against powdery mildew.*

Since CPM is one of the most destructive diseases affecting melon crops worldwide, the development of resistant melon varieties is a key strategy for sustainable disease management. Melon breeding programs have long sought to identify and harness genetic sources of resistance to powdery mildew. In total, 239 accessions have been described as resistant to different races (Cui *et al*., 2022) (**Table 2**).

Wild relatives of cultivated melon have proven to be invaluable reservoirs of resistance genes against CPM. Eleven years after race 1 was described for the first time, the resistant variety PMR 45 was released (Harwood & Markian, 1968; Kenigsbuch & Cohen, 1992). However, its resistance was broken only one year later due to the appearance of race 2 (Cui *et al*., 2022). Then, the variety PMR 5, which is resistant to both races 1 and 2, was developed and it was useful for 30 years, until race 3 appeared in 1976 (Epinat *et al.*, 1992; McCreight & Coffey, 2011). Ever since then, the number of emerging races has increased exponentially (**Table 1**). To this effect, the introgression of resistances to different races of CPM within a single

genetic background of melon is of great importance in breeding for durable resistance. This approach ensures that the melon crop remains protected even in the presence of diverse and rapidly evolving powdery mildew populations (Cui *et al*., 2022). Concretely, breeding efforts should be directed to the development of multi-resistant lines to control the predominant races in each region (McCreight *et al.*, 2012). However, out of the numerous accessions tested, only 13 have been confirmed to exhibit resistance against multiple CPM races (**Table 2**). Additionally, the identification of QTLs associated with these resistance responses remains limited, with only a few QTLs reported thus far (**Table 3**). In this sense, accessions PI 414723, PI 124112, TGR-1551, AR-5, WMR-29, VA-435 and PI 124111 are the most valuable resources to breed resistant melons, since QTLs ligated to the resistance to several races have been mapped. Nonetheless**,** in most cases the studies supporting the QTL detection are quite ancient and the resolution of the genetic maps is scarce. In this regard, in order to shorten the breeding programs, it would be important to take advantage of the cheapening of the new generation sequencing (NGS) technologies to fine map these QTLs and propose candidate genes for the resistance.

Among the described resistant cultivars, the Zimbabwean melon TGR-1551 exhibits resistance to races 1, 2, and 5 of *Px*. Inheritance studies were conducted by crossing TGR-1551 with the Spanish susceptible cultivar 'Bola de Oro,' revealing that resistance is controlled by two independent genes: one dominant and one recessive, exhibiting dominant-recessive epistasis (Yuste-Lisbona *et al*., 2010). A QTL analysis of the F_2 generation resulting from the initial cross identified a major QTL (*Pm-R*) on chromosome 5 associated with resistance to all three races (Yuste-Lisbona *et al*., 2011). Further evaluation of a RIL population (F7:F8) derived from the same cross confirmed the presence of the *Pm-R* QTL on chromosome 5, linked to the dominant gene, through QTL analyses performed by Beraldo-Hoischen *et al*. (2012). An additional QTL potentially associated with the recessive gene was identified for *Px* resistance on chromosome 12, with several microsatellite markers (TJ29, CMBR111, and CMBR150) reported to be associated with resistance (Beraldo-Hoischen *et al*., 2012). The recessive gene found in TGR-1551 is remarkable, as no recessive genes conferring resistance to multiple *Px* races have been reported in other melons accessions.

Table 2. List of melon accession that have been described as resistant to more than one powdery mildew race. The phenotype (S: susceptible; R: resistant) of each accession against the different races is indicated.

	***Podosphaera xanthii* races**																		
	1	**2**	**2F**	**2US**	**3**	**3.5**	**4**	**4.5**	**5**	**6**	**F**	**G**	**H**	**S**	**N1**	**N2**	**N3**	**N4**	**KN1**	**KN2**
VA-435	R	R	R		S	S	S	S	S					S						
AR-5	R	S	S		S	S	S	S	R					S	R					
TGR-1551	R	R			S	S	S	S	R											
PMR 45	R	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	R	R	S	S
PMR 6	R		R	R	S	R				R	S	S	S							
MR-1	R			R			R				S	R		S					R	R
PI 124111	R	R	R	R	S	R	R	S	S		S	R		S						
PI 313970	R	R	R	R	R	R	S	R	R					R						
PI 124112	R	R	S	R	R	S	R	S	S	R		R	S	S					R	R
WMR 29	R	R	R		R	S	S	S	S	S	S	S	R	S	R	R	R	R	S	S
PI 414723	R	R	R	S	R	R	R	R	R	R	S	S	S	S	S	S	S	S	S	S
EDISTO-47	R	R	R	S	S	R	R	R	S	S	S	S	S	S	R	R	S	S	S	R
PMR 5	R	R	S	R	S	S	R	S	R	R	S	S	S	S	R	R	R	R	S	R

Even though in other species the mechanisms of resistance can be classified as pre- and post-haustorial resistances (Gupta *et al.*, 1998; Kuzuya *et al.*, 2006; White & Baker, 1954), the first group, which is non-race specific, has not been observed in melon so far. Specifically, in relation to powdery mildew in melon, Kuzuya *et al*. (2006) distinguished two distinct post-haustorial resistance behaviors, namely type I and type II, which impede fungal development at different stages depending on the timing of the hypersensitive response (HR) in the epidermal cells. Type I resistance leads to the arrest of fungal growth at the primary hyphae stages, occurring around 48 hours post-inoculation (hpi), characterized by minimal callose accumulation in the penetration points and cell walls. This response has been observed for PMR 45, PMR 5 and PMR 6 genotypes. In contrast, type II resistance involves the occurrence of HR in the epidermal cells at a later time, resulting in more extensive fungal development within a greater number of cells, exhibiting callose accumulation around penetration points and cell walls. At 120 hpi, some conidiophores may also be observed. This kind of response has been observed in the genotype PI 414723. More recently, Beraldo-Hoischen *et al*. (2021) described two additional groups within the post-haustorial resistance, named IIa and IIb. Those accessions showing resistance mechanisms IIa and IIB have a different

behavior against CPM depending on temperature. This has been observed in TGR-1551. It has been hyphothesized that those genotypes carrying genes or QTLs controlling CPM resistance on chromosomes 5 and 9 would show a resistance type I. Whereas those with genes or QTLs located on chromosomes 2 or 12 would have a resistance type II (Beraldo-Hoischen *et al*., 2021). On the other hand, the occurrence of types IIa and IIb resistance behaviors in melon genotypes, characterized by multiple genes contributing to their powdery mildew resistance, suggests the potential involvement of epistatic interactions among these genes, which may account for the differential response to temperature (Beraldo-Hoischen *et al*., 2021).

A notable group of genes associated with powdery mildew resistance in melon is the *Mildew Resistance Locus O* (MLO) gene family. The MLO genes encode membrane proteins that play a crucial role in plant defense against powdery mildew pathogens, since they have been described as susceptibility factors (Iovieno *et al*., 2015; Zhang *et al*., 2021). *MLO* mutants are resistant to CPM because of the inhibition of the formation of haustrim in epidermal cells infected by the pathogen that did not die immediately (Aist & Bushnell, 1991; Koga *et al.*, 1990; Nelson *et al.*, 1990) . Moreover, the cell wall is thickened to resist the primary infection of CPM (Peterhänsel *et al.*, 1997). In melon, 14-16 MLO homolog genes, distributed in 5 different clades, have been identified (Iovieno *et al*., 2015; Zhang *et al*., 2021).Recently, 13 of these genes have been cloned and different expression patterns in resistant and susceptible genotypes were observed. As a summary, the expression assay results suggested that *CmMLO10* (Chr9: MELO3C021515) and *CmMLO5* (Chr10: MELO3C012438) could be involved in susceptibility to CPM, whereas *CmMLO8* (Chr7: MELO3C016709)*, CmMLO4* (Chr8: MELO3C007979)*, CmMLO13* (Chr11: MELO3C025761) and *CmMLO3* (Chr12: MELO3C005044) may also play a role in CPM resistance (Zhang *et al*., 2021). The sequence analysis of *CmMLO2* (Chr12: MELO3C005038) showed an 85 bp difference between wild and cultivated species (Iovieno *et al.*, 2015). Similarly, an SNP mutation in *CmMLO5* also resulted in loss of protein function in resistant accessions, thus resulting in CPM resistance (Zhang *et al*., 2021). This *CmMLO5* gene had been proposed by Cui *et al*. (2022) as a candidate gene derived from MR-1 conferring resistance against CPM race 1 (**Table 3**). Interestingly, there were no *CmMLO* members on chromosomes 1, 2, 4 and 5 (Zhang *et al*., 2021). This makes sense given that no QTLs have been mapped on chromosome 1, only a minor QTL mapped on chromosome 4 and the resistance genes mapped on chromosomes 2 and 5 show genetic control of resistance based on dominant genes (**Table 3**).

Table 3. Powdery mildew resistant accessions with characterized genetic control and mapped quantitative trait loci (QTLs). The tested races in the QTL analysis as well as the chromosome (chr) were the QTLs were mapped are indicated.

Resistance source	Races	Chr	*QTLs*	Genetic control	References
PI 414723	2, 2F	2	*Pm-x*	Dominant	(Périn *et al.*, 2002b)
	1, 5		*Pm-x1,5*		(Fazza *et al.*, 2013a)
	3		*Pm-x3*		(Fazza *et al.*, 2013a)
PI124112	1,2, 2F,3	5	*Pm-4, PmV.1*	Epistatic interaction	(Perchepied *et al.*, 2005)
	1, 2, 5	12	PmXII.1		
TGR-1551	1,2,5	5 12	*Pm-R*	Epistatic interaction	(Beraldo-Hoischen *et al.*, 2012; Yuste-Lisbona *et al.*, 2011)
AR-5	1, N1, 5	2 12	*QTL(AR5)*	Additive effect	(Fukino *et al.*, 2008a)
WMR-29	1, 2, 3	5 2	*Pm-w*	Dominant	(Périn *et al.*, 2002b) (Pitrat, 1991)
VA-435	1, 2, 2F	12	*Pm-Y*	Dominant	(Périn *et al.*, 2002b)
PI 124111	1, 2	7	*Pm-3*	Dominant for race 1, partially dominant for race 2	(Zhang *et al.*, 2013)
MR-1	1	10	*qPx1-10*	Recessive	(Branham *et al.*, 2021; Cui *et al.*, 2022)
		12	*qPx1-12,* BPM12.1	Dominant, epistatic effect	(Branham *et al.*, 2021; Cui *et al.*, 2022; Li *et al.*, 2017)
		5	*qPx1-5*	Major QTL, epistatic effect	(Branham *et al.*, 2021)
		4	*qPx1-4*	Minor QTL	(Branham *et al.*, 2021)
Edisto47	1B	2	*Pm-edisto47-1*	Dominant gene	(Ning *et al.*, 2014)
	1A	5	*Pm-Edisto47-2*		
PMR6	2F	2 12	*Pm2.1* *Pm12.1*	Epistatic effect	(Haonan *et al.*, 2020)
K7-1	2F	2	*Pm-2F*	Dominant	(Zhang *et al.*, 2013)
PI 78374	1	9	*Pm-1*	Dominant	(Teixeira *et al.*, 2008)
Ano2	1	5	*Pm-AN*	Single dominant gene	(Wang *et al.*, 2011)
wm-6		12	*qCmPMR-12*	Single recessive gene	(Cao *et al.*, 2021)
PI 134198	Ch1	12	*Pm-8*	Single dominant gene	(Liu *et al.*, 2010a)
PMR5	5	12		Single dominant gene	(Hong *et al.*, 2022)

Transcriptomic analyses using RNA sequencing (RNA-seq) have revealed specific defense mechanisms underlying powdery mildew resistance in melon (Cao *et al*., 2021; Polonio *et al*., 2019; Wang *et al*., 2021; Zhao *et al*., 2022; Zhu *et al*., 2018). P450 proteins and pathways related to phenylpropanoid biosynthesis may play important roles in *Px* resistance. Moreover, a wide transcriptional remodeling including seven functional groups (pathogen recognition, signal transduction, transcription factors (TFs), phytoalexin biosynthesis, other primary metabolite functions, MLOs and pathogenesis-related (PR) proteins) was also observed. Additionally, some lncRNAs (long non-coding RNAs) have been related to hydrolysis of chitin and callose deposition, as well as to stress-related terms (Gao *et al.*, 2020; Zhou *et al.*, 2020).

Further functional analysis should be conducted to validate the different candidate genes proposed to date. This information would be useful to develop functional markers linked to the resistance, which are more convenient for breeding purposes. Additionally, validated candidate genes also offer the possibility of generation transgenic or edited varieties resistant to CPM.

2.1.3. Melon breeding and its effects on fruit quality

The main drawback to the use of melon exotic germplasm with breeding purposes is that the introgression of the resistance regions could have pleiotropic effects on fruit quality. Fruit quality is a broad concept that includes a large number of characters, such as shape, size, color, texture, aroma or the nutritional content (Monforte *et al*., 2004; Paris *et al*., 2008). These traits have a complex genetic regulation, as they are usually controlled by multiple genes (Fernández-Trujillo *et al.*, 2011) and can also be highly influenced by environmental factors, such as the presence of pests and pathogens, the effect of grafting or the salinity of irrigation water (Cáceres *et al.*, 2017; Flores-León *et al.*, 2021). Moreover, the concept of fruit quality varies widely between groups and also depends on market demands. For example, fruits of the cantalupensis group should be aromatic, with a high sugar content, whereas those melons of the ibericus group have no aroma but contain a higher amount of sugars and also have a longer postharvest life than the cantalupensis group (Fernández-Trujillo *et al.*, 2011). What it is more, the same quality parameters could not be used for the different subgroups within the same group. If we look at the ibericus group, composed by Piel de sapo (PS), Amarillo, Branco, Rochet and Tendral subgroups, a great phenotypic variability can be observed for shape, size, color or organoleptic parameters (**Figure 5**). Thereby, fruit quality parameters should be set up for each group and subgroup individually.

However, as a consensus, to talk about melon fruit quality the following traits should be studied: external fruit characteristics (size, shape, rind color, rind firmness), internal characteristics (flesh color, size of seed cavity, flesh firmness, thickness of the flesh and internal rind), biochemical composition (content of acids, sugars, volatile aromatic compound (VOCs) and vitamins) and shelf-life (post-harvest life, cracking and vitrescence).

2.1.3.1. Fruit morphology.

Melon fruit morphology is a complex trait that includes both fruit shape (FS) and weight (FW). This character has been studied in different segregating populations and numerous QTLs ligated to this trait have been mapped (Castro *et al.*, 2019; Díaz *et al.*, 2017; Eduardo *et al.*, 2005; Harel-Beja *et al.*, 2010; Martínez-Martínez *et al.*, 2022; Monforte *et al.*, 2004; Oren *et al.*, 2020; Perpiñá *et al.*, 2016; Pereira *et al.*, 2018, 2021; Ramamurthy *et al.,* 2015; Santo Domingo *et al.*, 2022; Tomason *et al.*, 2013; Zalapa *et al.*, 2007). Interestingly,7 of those mapping populations were constructed with PS as recurrent parental (Castro *et al*., 2019; Díaz *et al*., 2017; Eduardo *et al*., 2005; Monforte *et al*., 2004; Pereira *et al*., 2018, 2021; Santo Domingo *et al*., 2022) and 3 of them used an accession of the agrestis group as donor parental (Díaz *et al*., 2017; Harel-Beja *et al*., 2010; Zalapa *et al*., 2007). Recently, Pan *et al*. (2020) stablished 78 consensus QTLs related to fruit morphology from different works. Additionally, 87 homologous genes from 8 genetic families (CNR (cell number regulator), CSR (cell size regulator), CYP78A (cytochrome P450), SUN, OVATE, TRM (TONNEU1 Recruiting Motif), YABBY and WOX) were also related to this character. More recently, an increase in the expression level of a member of the Ovate Family Proteins (*CmOFP13,* chr 8, MELO3C025206) has been identified as the causal agent of the round shape (Martínez-Martínez *et al.*, 2022).

As for fruit weight, QTLs have been detected in almost all the chromosomes with different genetic backgrounds (Castro *et al*., 2019; Eduardo *et al*., 2005; Harel-Beja *et al*., 2010; Monforte *et al*., 2004; Oren *et al*., 2020; Pereira *et al*., 2018; Perpiñá *et al*., 2016; Zalapa *et al*., 2007). FW is correlated with both fruit length (FL) and diameter (FD). Numerous QTLs have also been associated to FL but those located in chromosomes 2 and 8 have been detected in different genetic backgrounds (Eduardo *et al.*, 2005; Harel-Beja *et al.*, 2010; Perpiñá *et al.*, 2016; Díaz *et al.*, 2017; Pereira *et al.*, 2018; Castro *et al.*, 2019) with introgressions of the agrestis group in these QTL producing more elongated fruits. As for FD, different QTLs have been mapped on most of the chromosomes (Castro *et al.*, 2019; Harel-Beja *et al.*, 2010; Perpiñá *et al.*, 2016; Zalapa *et al.*, 2007).

Monforte *et al*. (2014) and Gonzalo & Monforte, (2017) defined 4 Meta-QTLs for FW on chromosomes 2,3,8 and 11 and 5 additional Meta-QTLs on chromosomes 1, 2, 8, 11 and 12 for FS. Most of the later QTLs co-localized with those previously detected for FL and FS. Moreover, orthologous genes to those of SlKLUH/FW3.2 of tomato could be related to variations in FW, as genes of the family *OVATE* are associated to FS (Monforte *et al*., 2014; Pan *et al*., 2020).

2.1.3.2. External fruit characteristics.

One of the most important traits that influences costumers is the rind color. Carotenoids (β-carotene), chlorophylls and flavonoids (naringenin chalcone) are responsible of orange, green and yellow colors of the rind, respectively (Burger *et al.*, 2006; Tadmor *et al.*, 2010). The gene *CmKFB* (Chr10: MELO3C011980, Kelch domain-containing F-Box protein) negatively regulates the accumulation of naringenin chalcone, altering the metabolic flux of flavonoids (Tadmor *et al.*, 2010; Feder *et al.*, 2015). Additionally, Pereira *et al*. (2018) detected 3 QTLs linked to rind yellowing on chromosomes 5, 10 (*CmKFB*) and 12, and the QTL on chromosome 10 was also detected by Castro *et al*. (2019). It has been proposed that the QTLs that mapped on chromosomes 5 and 10 could epistatically regulate the flavonoids synthesis.

Other major genes related to the external fruit characteristics, such as stripped epicarp (gene *st,* chromosome 3), spots on the rind (gen *mt*-2, chromosome 2) and sutures (gen *s-2,* chromosome 11) have also been mapped (Danin-Poleg *et al.*, 2002; Périn *et al.*, 2002b). Moreover, rind netting QTLs have been mapped on chromosomes 2, 9, 11 and 12 (Harel-Beja *et al.*, 2010; Oren *et al.*, 2020).

2.1.3.3. Internal fruit characteristics.

Regarding the fruit internal appearance, an important trait is to have small cavities, so that the total flesh content is increased. This characteristic is controlled by a major gene named *Empty cavity, Ec,* which has been mapped on chromosome 2 (Périn *et al*., 2002a). Additional QTLs linked to cavity diameter were mapped on chromosomes 1, 2, 5 and 11 (Castro *et al.*, 2019; Paris *et al.*, 2008). Other traits that influence the internal appearance of melon include rind thickness and skin firmness which are positively correlated with postharvest life and storage. Obando *et al*. (2008) identified a QTL on chromosome 12 associated with this trait, while Castro *et al*. (2019) mapped several QTLs linked to rind thickness on chromosomes 3, 4, 6 and 7. As for flesh firmness, it is an ethylene-dependent characteristic that can determine the fruit quality of melon (Pech *et al.* 2008) and regions linked to this trait have been mapped on chromosomes 2, 3, 5, 6, 7, 8, 10 and 12 (Dai *et al.*, 2022;

Chen *et al.*, 2023; Moreno *et al.*, 2008; Oren *et al.*, 2022; Paris *et al.*, 2008; Perpiñá *et al.*, 2016).

Another important character is the color of the flesh. This trait, which is independent of the production of ethylene (Flores *et al*., 2001), is controlled by two major genes with an epistatic interaction (Clayberg, 1992): *green flesh* (*gf*) (Hughes, 1948) and *white flesh* (*wf*) (Iman *et al.*, 1972). On the one hand, *gf* (chr 9, MELO3C005449) induces β-carotenoids accumulation (Burger *et al*., 2010; Diao *et al*., 2023; Tzuri *et al*., 2015). On the other hand, *wf* has been mapped on chromosome 8 (Harel-Beja *et al*., 2010; Monforte *et al*., 2004). This *locus* has been associated with *CmPPR1* (MELO3C003069) (Galpaz *et al.*, 2018) and MELO3C003097 (Zhao *et al*., 2019).

2.1.3.4. Fruit ripening.

Depending on their ripening behavior, melons have typically been divided into climacteric (i.e. cantalupensis group) and non-climacteric (i.e. ibericus group). Climacteric fruits undergo a ripening stage characterized by a rapid increase in respiration and ethylene production. This phase is associated with significant changes in color, texture, flavor, and aroma (Pech *et al.*, 2008). Climacteric fruits can continue the ripening process even after being harvested. In contrast, non-climacteric varieties do not exhibit a similar peak in respiration and ethylene production during ripening. Non-climacteric fruits have a limited ability to continue ripening after harvest and often reach their optimal quality while still attached to the plant. However, a great variability between climacteric and non-climacteric melons have been observed (Ezura & Owino, 2008; Pereira *et al*., 2018; Perpiñá *et al*., 2016; Saladié *et al*., 2015). Multiple studies have been conducted to analyze this trait and QTLs have been mapped on chromosomes 1, 2, 3, 6, 7, 8, 9, 10 and 11 (Périn *et al.*, 2002a; Moreno *et al.*, 2008; Perpiñá *et al.*, 2016; Ríos *et al.*, 2017). Moreover, recent studies propose that non-climacteric fruits may harbor mutations in genes involved in ethylene biosynthesis and signaling or in transcription factors similar to those defined in tomato, such as CNR, NOR, and RIN (Giovannoni, 2004; Saladié *et al.*, 2015). QTLs related to ethylene emission, fruit earliness and other ripening related traits, such as rind firmness, have recently been mapped on chromosomes 3 and 8 (Oren *et al.*, 2022). This work proposed MELO3C011432 (chr 3, WRKY family transcription factor) and MELO3C011365 (chr 3, transducing/WD40 repeat-like superfamily protein) and MELO3C007661 (chr 8, transmembrane protein putative gene) as candidate genes responsible of fruit ripening.

2.1.3.5. Sugars and organic acids profile.

The existing variability in sugar content in melon is quite extensive, ranging from 3 to 5 °Brix in non-sweet melons to 10 to 18 °Brix in sweet melons (Burger *et al*., 2010; Stepansky *et al*., 1999). The accumulation of sugars is an independent trait from ethylene production (Flores *et al*., 2001; Pech *et al*., 2008). The three main sugars in melon pulp are glucose, fructose, and sucrose, with sucrose contributing the most to the sweetness of the melon (Stepansky *et al.*, 1999). The increase in total sugars during fruit ripening is mainly attributed to sugar accumulation during the final stage of fruit development (Gur *et al.*, 2017). Saladié *et al*. (2015) observed that, for both climacteric and non-climacteric varieties, the evolution of these sugars was similar. The levels of fructose and glucose did not show significant variation during fruit development and ripening, whereas sucrose levels increased as the fruit began to ripen. The accumulation of sucrose was controlled by a major recessive gene, sucrose (*suc*) gene, present in sweet melon varieties, although its genetic control appears to be complex, involving other *loci*. However, this trait is difficult to evaluate due to its strong environmental influence (Burger *et al.*, 2002; Perpiñá *et al.*, 2016). The main enzymes related to sucrose metabolism include sucrose synthase (SS), sucrose phosphate synthase (SPS), invertases (INV), and α-galactosidases (AGAL) (Burger & Schaffer, 2007; Dai *et al*., 2011). Understanding the balance between sucrose breakdown activities (INV and SS) and sucrose synthesis (SPS) determines the net accumulation of sucrose (Burger & Schaffer, 2007).

Numerous studies have identified genomic regions associated with sugar content in melon. Monforte *et al*. (2004) mapped QTLs on chromosomes 1, 2, 4, and 8. Subsequently, Eduardo *et al*. (2007) observed 19 NILs (nearly isogenic lines) with significant differences compared to the recurrent parent, of which two showed alterations in soluble solids content. The introgressions of these lines were located on chromosomes 1 and 3. Similarly, Obando-Ulloa *et al*. (2009) detected a series of QTLs on chromosomes 3, 5, 6, 7, and 11 affecting soluble solids content. In other genetic backgrounds, Paris *et al*. (2008) detected QTLs on chromosomes 1, 2, 6, 7, 8, 9, and 10 affecting soluble solids content. On the other hand, Park *et al*. (2009), using the parents 'Deltex' (ameri group) x TGR 1551 (acidulus group) in an F_2 population, observed QTLs on chromosomes 2, 3, 4, 6, and 11 related to soluble solids content, sucrose, fructose, and glucose. Perpiñá *et al*. (2016) mapped QTLs linked to sugars content on chromosomes 1, 5, 10 and 11 and Castro *et al*. (2019) found QTLs linked to this trait on chromosomes 2, 4, 5, and 7. Due to the extensive genetic variability in sugar content, Leida *et al*. (2015) described the genetic structure of 175 melon accessions, representing the phenotypic variability of the

species, and performed an association analysis between polymorphisms and sugar content. Recently, Argyris *et al*. (2017) used several populations generated from PS and 'Ames 24294' (*C. melo,* agrestis group) to analyze QTLs related to sugar and organic acid content, mapping QTLs on chromosomes 1, 2, 3, 4, 5, 6, 7, and 12 related to sugar content, on chromosomes 2, 3, 4, 5, and 8 related to sucrose content, on chromosomes 5, 6, 7, 8, and 11 related to fructose content, and on chromosomes 4, 5, 6, 7, 8, and 11 related to glucose content. Lastly, Pereira *et al*. (2018) detected QTLs affecting soluble solids content on chromosomes 8, 9, and 10. There is a complex genetic control of sugar accumulation, and transcriptomic studies have revealed the interaction of a broad network of 50 genes involved in sucrose accumulation (Dai *et al*., 2011; Zhang *et al*., 2016). However, some regions consistently appear, such as the QTL located on chromosome 5, which remains stable across studies, years, and genetic backgrounds (Castro *et al.*, 2019; Eduardo *et al.*, 2007; Monforte *et al.*, 2004; Obando-Ulloa *et al.*, 2009; Paris *et al.*, 2008; Perpiñá *et al.*, 2016;). MELO3C014519 (BEL1-like homeodomain protein) is located within this region and is considered a promising candidate associated with sugar accumulation.

Acidity, along with sweetness, is a characteristic that determines melon quality. The flexuosus and acidulus groups are the most acidic compared to sweet melons (Gonzalo & Monforte, 2017). Acidity is controlled by the dominant gene *So* (*Sour*), located on chromosome 8, and its expression is ethylene independent (Burger *et al.*, 2002; Harel-Beja *et al.*, 2010). The gene responsible for *So*, *CmPH* (MELO3C025264, auxin efflux carrier family protein), was recently cloned (Cohen *et al*., 2014). Melon acidity is largely attributed to malic and citric acids. A recent study detected QTLs on chromosomes 2, 8, and 11 affecting malic acid content and on chromosomes 8 and 10 affecting citric acid content (Argyris *et al.*, 2017). Park *et al*. (2009) mapped a QTL linked to abscorbic acid accumulation on chromosome 5.

2.1.3.6. VOCs.

Aroma is one of the key traits that determine melon quality (Beaulieu & Lea, 2006; Fallik *et al.*, 2001; Kourkoutas *et al.*, 2006), and it is closely related to flavor (Flores-León *et al.*, 2022; Lignou *et al.*, 2014; Obando-Ulloa *et al.*, 2010; Tang *et al.*, 2015;). Both aroma and flavor are often attributed to complex blends of volatile organic compounds (VOCs). In melon, more than 300 volatile compounds have been detected (Gonda *et al.*, 2013). Freilich *et al*. (2015) investigated the relationship between sugar content, color, and aromas during fruit ripening using metabolic and transcriptomic analyses in an RIL population derived from the 'Dulce' variety and the PI 414723 accession. They concluded that traits related to sugar content and

color are ethylene-independent, whereas the production of volatile compounds is ethylene-dependent. The main volatile compounds in climacteric fruits are esters and alcohols, whereas in non-climacteric fruits, aroma is determined by low levels of these compounds and the presence of aldehydes, organic acids, and terpenes (Shalit *et al.*, 2001).

2.2. Genetic and genomic tools for melon breeding.

To achieve successful introgression of desirable traits, breeders employ various strategies such as conventional breeding, molecular marker-assisted selection, and advanced genomic techniques. These approaches facilitate the identification and transfer of resistance genes from wild relatives, enabling the development of improved melon cultivars with enhanced resistance to pathogens.

2.2.1. Genomic resources.

In cucurbits breeding, different strategies have been developed to facilitate the selection and introgression of agronomic traits of interest, such as disease resistance, fruit quality or crop yield (Hao *et al.*, 2020). The combination of classical breeding techniques with novel molecular, genomic and bioinformatic tools has been a revolutionary advance in recent decades, lowering costs and increasing the efficiency of breeding programs (Grumet *et al.*, 2017).

Until ~10 years ago, the study of melon genetics was limited to the use of molecular markers, such as SSRs (simple sequence repeats), CAPs (cleaved amplified polymorphic sequence), AFLPs (amplified fragment length polymorphisms) and RAPDs (random amplified polymorphic DNA), that were linked to interesting agronomical traits. The use of these molecular markers was time consuming, and the resolution and saturation of the genetic maps obtained was scarce, which limited the applicability of the obtained results (Diaz *et al.*, 2011). Moreover, even though multiple genetic maps were available, the lack of common molecular markers between them made it difficult to compare results obtained from different assays. The first consensus map for molecular markers in melon was constructed by Diaz *et al*. (2011) and it included 1592 markers (640 SSRs, 330 SNPs (single nucleotide polymorphism), 252 AFLPs, 239 RFLPs (Restriction fragment length polymorphism), 89 RAPDs, 15 IMAs, 16 indels and 11 morphological traits.

All this changed when the first version of the melon genome (v.3.5) was released on 2012 (Garcia-Mas *et al.*, 2012). Melon has genome size of 357.64 Mb (x=12) (Garcia-Mas *et al.*, 2012), which is big compared to other cucurbits species such as watermelon (425 Mb, x=11) (Guo *et al.*, 2013) or cucumber (367 Mb, x=7) (Huang *et al.*, 2009) but it is relatively small when compared to other agronomically

important species like potato (*Solanum tuberosum,* 844 Mb, x=12) (Visser *et al.*, 2009) , tomato (*Solanum lycopersicum,* 900 Mb, x=12) (Sato *et al.*, 2012), pepper (*Capsicum annum,* 3480 Mb, x=12) (Kim *et al*., 2014) or wheat (*Triticum aestivum,* 17000 Mb, x=7) (Appels *et al.*, 2018).

The first version of the melon genome included 27.427 annotated protein-coding genes, with 17% of the genome being transposable elements. Then an improved assembly (v.3.5.1) was obtained using a shotgun strategy with 454 sequencing. This new assembly covered 83.3% of the genome (375 Mb). In 2018, version 3.6.1 was released. This version was improved using optical mapping, and a new annotation was built, including 29.980 protein-coding loci. The latest version of the genome (v.4.0.) was created using PacBio single-molecule real-time (SMRT) sequencing. The DHL92 melon assembly v4.0 exhibited a notable expansion in the size of the melon genome pseudomolecule, with an increase of 40 Mb compared to v3.5.1. Furthermore, it demonstrated substantial improvement in gap filling, with 90% of the v3.5.1 gaps now filled. This advancement can be attributed to the enhanced capabilities of transposable element (TE) annotation tools, resulting in an increase in TE coverage from 19.7% (v3.5.1) to 45.2% (v4.0).

In addition to the reference genome based on the genotype DHL92 (a double haploid line derived from an inodorus x agrestis cross), other 5 additional reference genomes are available in the Cucurbit genomics database (http://cucurbitgenomics.org/) (**Table 4**). These genomes were also generated using third generation sequencing technologies, such as PacBio and Oxford nanopore, and were obtained from accessions belonging to the cantalupensis, inodorus, agrestis and reticulatus groups and there is also available one melon from an accession of *C. metuliferus.*

The availability of these genomes has contributed to the development and expansion of molecular markers collections, enriching mapping populations for genetic analysis of various traits and making it possible to locate and identify genes of interest (Hao *et al*., 2020). In addition, the lower costs of NGS technologies have facilitated the massive resequencing of large collections of melon accessions, improving our knowledge of the overall diversity within this species (Guo *et al*., 2019b; Wu *et al*., 2019; Zhao *et al*., 2019).

Table 4. List of the melon reference genomes available.

Species	Genotype used	Version	Assembled genome size	Protein-coding genes	Sequencing technology	Reference
C. melo, DH, ibericus x conomon	DHL92	3.5	375 Mb	27.427	454 reads and Sanger sequencing	García-Mas *et al*. (2012)
C. melo, DH, ibericus x conomon	DHL92	3.5.1				Argyris *et al*. (2015)
C. melo, DH, ibericus x conomon	DHL92	3.6.1	375.36 Mb	29.980	Optical mapping approach	Ruggieri *et al*. (2018)
C. melo, DH, ibericus x conomon	DHL92	4	357.64 Mb	28.299	PacBio and Illumina sequencing	Castanera *et al*. (2020)
C. melo, cantalupensis group	Charmono	1.1	365 Mb	31.348	PacBio combined with Hi-c and Illumina	Pichot *et al*. (2022)
C. melo, inodorus group	Payzawat	1	386 Mb	22.924	PacBio combined with the Hi-c interaction analysis	Zhang *et al*. (2019)
C. melo, agrestis group	IVF77	1	364 Mb	27.073	PacBio SMART platform and Illumina sequencing	Ling *et al*. (2021)
C. melo, reticulatus group	Harukei-3	1.41	378 Mb	33.829	Oxford nanopore	Yano *et al*. (2020)
C. metuliferus	PI 482460	1	329 Mb	29.039	PacBio SMART platform and Illumina sequencing	Ling *et al*. (2021)

Remarkably, already in the first version of the genome it was observed that there was a low number of nucleotide-binding site-leucine-rich repeat (NBS-LRR) disease resistance genes annotated (Garcia-Mas *et al.*, 2012). Plant disease resistance NBS-LRR genes are pivotal in the recognition of proteins encoded by pathogen-specific avirulence genes (Jones & Dangl, 2006). The abundance of NBS-LRR genes in various plant genomes exhibits a remarkable range, with some genomes harboring fewer than 100 genes (e.g., papaya and cucumber), while others contain over 1000 genes (e.g., wheat) (Ming *et al.*, 2008; Huang *et al.*, 2009; Jia *et al.*, 2013). When the genome of *C. metuliferus* was compared to those of melon and cucumber it was observed that this wild-type relative has a greater number of resistance genes than the cultivated cucurbits. The depletion of NBS-LRR *loci* in *Cucurbitaceae* can be

attributed to the occurrence of large insertions or deletions (indels) and the pseudogenization resulting from small indels, which collectively account for the loss of NBS-LRR genes in this plant family (Ling *et al.*, 2021). In melon, only 15 genes have been annotated as NBS-LRR proteins, which suggests the existence of specific defense mechanisms in this species.

2.2.2. SNPs genotyping methods.

Different types of molecular markers have been used for melon genetic analysis: RFLPs, RAPDs, SSRs and SNPs. Among them, SNPs represent the most frequent type of DNA variation in the genome (Wang *et al*., 1998), since they are polymorphisms that affect a single nucleotide by a substitution, an insertion or a deletion. These markers are codominant, reproducible and mostly biallelic and can be easily identified *in silico* by using a reference genome and sequencing data. The automation of SNPs detection through the use of medium- and high-throughput platforms has enabled the characterization of large collections and the saturation of genetic maps, which has been translated into great advances in molecular plant breeding. Among these genotyping platforms Agena Bioscience iPLEX ® Gold MassARRAY (former Sequenom; Gabriel *et al*., (2009)), Illumina´s GoldenGate technology (Fan *et al.*, 2006), the genotyping by sequencing (GBS) technique (Elshire *et al.*, 2011) and DArTseq genotyping should be highlighted. Moreover, transcriptomic data has also been used to detect new SNPs variants not previously detected (Blanca *et al*., 2012; Blanca *et al*., 2011)

To genotype a sample through GBS an enzymatic restriction of the genome is carried out, which reduces its complexity and generates a representative library of the complete genome, that is sequenced (Elshire *et al.*, 2011). This allows the identification of a high number of SNPs distributed throughout the genome. GBS has been used to study the genomic variability of melon as well as to fine map interesting traits (Flores-León *et al*., 2022; Gonzalo *et al*., 2019; Pavan *et al*., 2017; Pereira *et al*., 2018; Pérez-de-Castro *et al*., 2019). A cheaper alternative to GBS, also based on the use of restriction enzymes and sequencing is DarTseq. This technique enriches the libraries in predominantly active – low copy sequence- areas in a genome and has also been used to study cucurbits populations (Yang *et al.*, 2016; Abu Zaitoun *et al.*, 2018).

When collections of SNPs derived from sequencing data are available and medium or small-scale genotyping is required, platforms such as Agena Bioscience iPLEX ® Gold MassARRAY can be used. This technology conducts a multiplex PCR followed by mass spectrometry to distinguish between the alleles of a *loci* (Gabriel *et al.*, 2009). This platform has widely been used to map QTLs ligated to interest traits in

different melon populations (Esteras *et al.*, 2013; Perpiñá *et al.*, 2016; Castro *et al.*, 2019). SNPs can also be detected by high resolution melting (HRM) or competitive allele-specific PCR (KASP), which are both also based on a PCR reaction. HRM conducts a real-time PCR and the allele of the targeted SNP is identified based on the emitted fluorescence and denaturation temperature (Vossen *et al.*, 2009). On the other hand, KASP relies on the end-point fluorescent read (Semagn *et al.*, 2014). Both types of markers have been widely used to genotype melon populations (Branham *et al.*, 2021; Cao *et al.*, 2021; Castro *et al.*, 2019; Perpiñá *et al.*, 2016; Sáez *et al.*, 2020).

2.2.3. Mapping populations and QTL analysis.

Mapping populations and marker-assisted selection (MAS) have emerged as valuable tools for breeding programs in melon. Mapping populations provide the necessary genetic diversity for trait mapping and identification of quantitative trait k (**Figure 6**). These populations enable the construction of genetic maps, facilitating the localization of genomic regions associated with desirable traits. A wide range of mapping populations have been used to map resistance traits in melon: F_2 populations (Danin-Poleg *et al*., 2002; Herman & Perl-Treves, 2007; Kawazu *et al*., 2018; Li *et al*., 2017; Sáez *et al*., 2017; Wang *et al*., 2016; Yuste-Lisbona *et al*., 2011), RILs (Branham *et al.*, 2018, 2021; Palomares-Rius *et al.*, 2011; Perchepied *et al.*, 2005; Pérez-de-Castro *et al.*, 2019) , ILs (isogenic lines) (Areco & Martín-Hernández, 2022), NILs (Essafi *et al.*, 2009; Guiu-Aragonés *et al.*, 2014), DHL (double haploid lines) (Guiu-Aragonés *et al.*, 2014) and backcrosses (BC) (Danin-Poleg *et al.*, 2002; Herman & Perl-Treves, 2007; Teixeira *et al.*, 2008; Sáez *et al.*, 2017). The genotyping and phenotyping of these mapping populations has allowed the detection of QTLs linked to resistances.

The use of a set of molecular markers evenly distributed throughout the genome can speed the development of these mapping populations as well as breeding programs, by facilitating the selection of those lines carrying a lesser amount of the donor parental genome (Castro *et al*., 2019; Pereira *et al*., 2021; Perpiñá *et al*., 2016; Santo Domingo *et al*., 2022). Altogether, the use of both molecular markers tightly linked to the resistance QTLs and background selections accelerates the breeding process, increases selection accuracy, and enables the introgression of desirable traits from wild or unadapted germplasm into elite melon varieties. Thus, the integration of mapping populations and MAS offers significant potential to enhance the efficiency and effectiveness of melon breeding programs, enabling the development of improved varieties with desired traits.

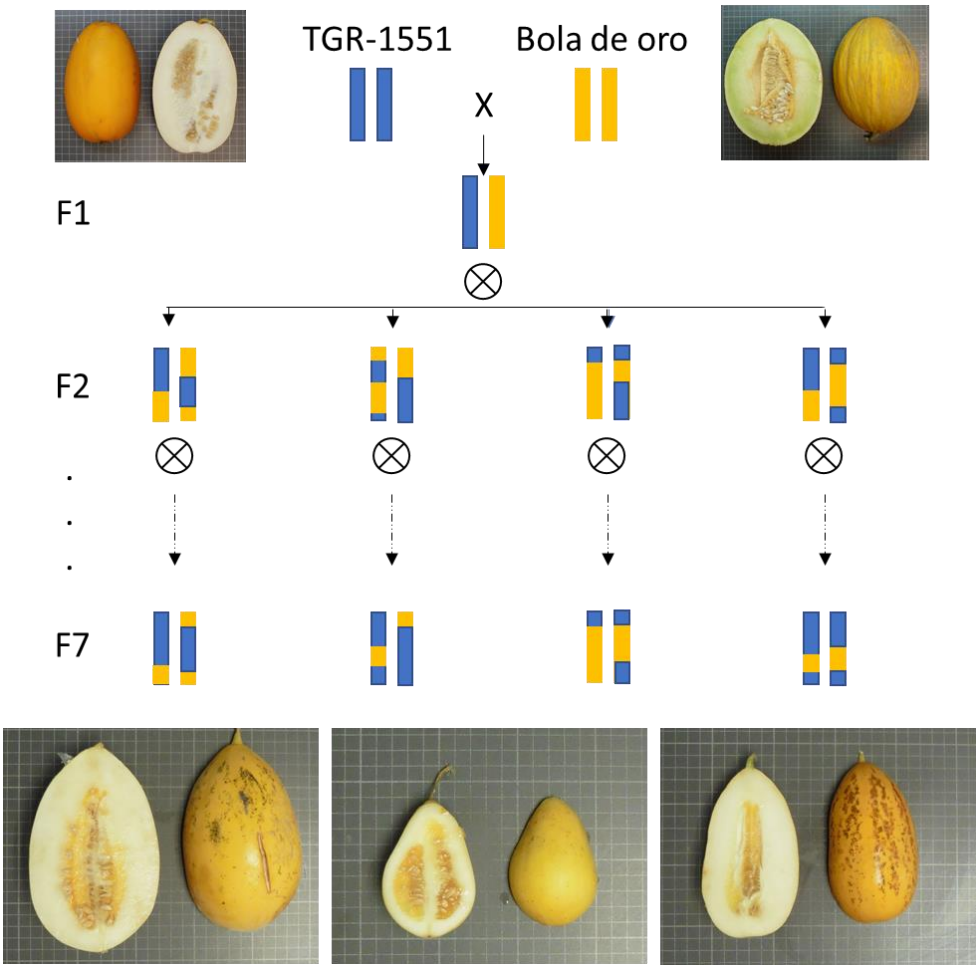

Figure 6. Development of a RILs (Recombinant Inbred Lines) population derived from the initial cross between TGR-1551 and the Spanish cultivar `Bola de Oro´.

2.2.4. Reverse genetics platforms.

TILLING (Target Induced Local Lesions in Genoms) and ecoTILLING (Eco-type TILLING) have been used in cucurbits to study the function of mutated genes, either by natural or induced variation. These kinds of platforms are especially useful when no efficient transformation protocols are available. In melon, they have been used to study the sexual determination of melon (Boualem *et al.*, 2015), to identify an allelic variant that confers resistance to MNSV (Nieto *et al.*, 2007) and to validate a candidate gene (*Vacuolar Protein Sorting 41, CmVPS41*) conferring resistance to CMV in melon (Giner *et al.*, 2017).

2.2.5. Transcriptomic analysis.

In addition to the previously listed reference genomes, the transcriptome of melon is also available (Blanca *et al*., 2011). Transcription analysis can be used to better understand the biological function, implication in different metabolic routes and cellular processes of candidate genes linked to different agronomical traits.

Transcriptomic analyses were firstly carried out by using microarrays platforms. This technology is based on the hybridization of RNA with different probes labeled with fluorophores. Microarray experiments conducted in melon have provided valuable insights into gene expression patterns and regulatory networks associated with various biological processes. For example, it has been used to study the transcriptomic changes produced by CMV and MNSV infection in susceptible cultivars (Gómez-Aix *et al.*, 2016; Mascarell-Creus *et al.*, 2009), and also to compare the differential response of susceptible and resistant genotypes inoculated with *M. cannonballus* and WMV (Gonzalez-Ibeas *et al.*, 2012; Roig *et al.*, 2012).

However, the cheapening of NGS technologies has popularized the use of RNA-seq technology to study transcription changes in melon. RNA-seq presents several advantages over microarrays. Firstly, it offers enhanced coverage of the transcriptome (Sekhon *et al.*, 2013). Unlike microarrays, which are limited to the annotated genes available at the time of the experiment, RNA-seq enables a more comprehensive exploration of all transcriptional changes induced by a stimulus (Sekhon *et al.*, 2013; Cervantes-Gámez *et al.*, 2016). Additionally, RNA-seq provides more accurate measurements of gene expression by directly counting reads, surpassing the limitations of microarray-based measurements (Marioni *et al*., 2008; Ozsolak & Milos, 2011). In summary, RNA-seq technology proves to be more valuable than microarrays for discovering novel transcriptional features, identifying differentially expressed genes (DEGs), and detecting alternative gene splicing. This technique has recently been used to study transcriptional changes in cucurbits during viral infections (Amoroso *et al.*, 2021; Li *et al.*, 2017; Lou *et al.*, 2020; Sáez *et al.*, 2022a; Sun *et al.*, 2017). Moreover, numerous RNA-seq assays have been conducted to study the differential response of resistant and susceptible melon genotypes against fungal infections (**Table 5**). However, to our knowledge, only one RNA-seq study has been performed to study the response against viruses (ToLCNDV) in melon (Sáez *et al*., 2022a).

Table 5. List of RNA-seq assays carried out in melon to detect differentially expressed genes related to defense against pathogens.

Pathogen	Transcript studied	Genotypes	Phenotype	Time points	Nº DEGs at each time point	Reference
Powdery mildew	mRNA	MR-1 Topmark	Resistant Susceptible	0, 24, 72 and 168 hpi	561, 693 and 1506 1239, 2144 and 1579	(Zhu *et al*., 2018)
Powdery mildew	mRNA	Rochet	Susceptible	24, 48 and 72 hpi	1114, 3785 and 4226	(Polonio *et al.*, 2019)
Powdery mildew	mRNA	Yangjiaomi	Susceptible	9 dpi	4808	(Wang *et al*., 2021)
Powdery mildew	mRNA	WM-6 12D-1	Resistant Suceptible	3 dpi	3000	(Cao *et al.*, 2022)
Powdery mildew	mRNA	TG-1 TG-5	Resistant Susceptible	0, 1,2,3 and 5 dpi	789, 246, 166 and 459 1469, 3034, 1080 and 738	(Zhao *et al*., 2022)
Powdery mildew	mRNA	GanTianmi XueLianHua	Resistant Susceptible	-	1166 1147	(Hong *et al*., 2019)
Powdery mildew	lncRNAs	MR-1 Top Mark	Resistant Susceptible	0, 24, 72 and 168 hpi	254	(Zhou *et al.*, 2020)
Powdery mildew	lncRNAs	M1 B29	Resistant Susceptible	0, 24 and 48 hpi	387 183	(Gao *et al.*, 2020)
F. oxysporum	mRNA	NAD CHT	Resistant Susceptible	24 and 48 hpi	2461 and 821 882 and 2237	(Sebastiani *et al.*, 2017)
P. capsici	mRNA	ZQK9 E31	Resistant Susceptible	0, 3 and 5 dpi	178 and 1064 3792 and 4642	(Wang *et al*., 2020)
ToLCNDV	mRNA	WM7 Piñonet (PS)	Resistant Susceptible	0, 3, 6 and 12 dpi	292, 354, 558 404, 752, 981	(Sáez *et al.*, 2022a)

2.2.6. Genetic engineering and genome editing: transgenics and CRISPR/Cas9.

Transgenesis and genome editing have emerged as an efficient alternative to classic breeding programs to obtain resistant plants to both biotic and abiotic stresses. For example, a common technique to create resistant varieties against viral infections consists in the silencing of genes that the virus needs to complete its life cycle in the plant. For this purpose, artificial microRNAs (amiRNAs) that encode a non-translatable sequence of the virus genome are designed. The virus coat protein is the most common transgene to generate virus-resistant plants (Ram *et al.*, 2019). This strategy has been used to obtain melon plants with CMV resistance (Yoshioka *et al.*, 1993).

CRISPR/Cas9 technology has also been used to generate resistant melons. *Eif4e* knockout melon mutant lines were resistant to MWMV but also showed male sterility (Pechar *et al.*, 2022). Genome editing has also recently been used to functional validate candidate resistance genes against PRSV and *Fusarium oxysporum* f.sp. *melonis* (Nizan *et al.*, 2023). However, CRISPR/Cas9 has not been widely used for genome editing and gene validation in cucurbits due to the regeneration difficulties derived from the *Agrobacterium tumefaciens* transformation. Moreover, to date, the legal procedures to commercialize genetically modified products and the current unfavorable social convictions against them prevent their acceptance and reduce the practical value of these techniques, especially in the European Union. Nevertheless, it seems that this trend could be reversed in the coming years, which would lead to greater investments for the development and applicability of this technique in melon.

Objectives

Objectives

Viral and fungal diseases are the main limiting factors for the cultivation of cucurbits. Breeding resistant varieties against these pathogens is the most effective, durable, and environmentally friendly strategy to fight this threat. To conduct effective breeding programs, it is necessary to study the predominant pathogens in the main producing areas, as well as to decipher the genetic basis associated to the resistances against them.

The general objective of the present Ph.D. dissertation was the generation of information and tools for the development of a breeding program to introgress the resistances to cucurbits yellows stunting disorder virus (CYSDV), watermelon mosaic virus (WMV) and cucurbits powdery mildew (CPM) races 1, 2 and 5, derived from the African accession TGR-1551, into commercial melon genetic backgrounds. In order to achieve this goal, four sub-objectives have been addressed, which frame the five chapters in which this thesis is structured.

Sub-objective 1: To identify and to study the genetic diversity of the main viruses affecting cucurbits production in the Spanish Mediterranean basin.

Sub-objective 2: To fine-map the resistances against CYSDV and CPM derived from TGR-1551 in order to obtain molecular markers tightly linked to the resistance regions.

Sub-objective 3: To characterize the transcriptomic changes taking place during WMV infection in the susceptible cultivar `Bola de Oro' and in a TRG-1551-derived line, in order to identify candidate genes associated to WMV resistance derived from TGR-1551.

Sub-objective 4: To develop a breeding program aimed to introgress the resistances to CYDV, WMV and CPM into commercial melon genetic backgrounds.

Results

Chapter 1.

Incidence and genetic diversity of cucurbit viruses in the Spanish Mediterranean area

Chapter 1. Incidence and genetic diversity of cucurbit viruses in the Spanish Mediterranean area

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https://drive.google.com/drive/folders/1OQsVbVaDPjsoBWgpSkd4EqFmtwodcfuU?usp=sharing

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Abstract

Viral infections on cucurbits fields can cause major economic losses. Monitoring of the main producing areas is essential to identify both prevalent and emerging viruses. For two consecutive years (2019-2020), the presence and molecular diversity of 9 aphid- and whitefly-transmitted viruses in the main cucurbits producing areas of the Spanish Mediterranean basin and other important regions were studied. In analyses of symptomatic plants, watermelon mosaic virus (WMV), cucurbit aphid-borne yellows virus (CABYV) and cucumber mosaic virus (CMV) were found to be prevalent in all the monitored areas, regardless of the crop and the farming conditions. Moroccan watermelon mosaic virus (MWMV) and tomato leaf curl New Delhi virus (ToLCNDV) were also found at lower rates, mainly in mixed infections with WMV. Phylogenetic analyses were conducted to determine the molecular variability of the different isolates. Whereas the sequences of CABYV, MWMV and ToLCNDV isolates all clustered within their corresponding Mediterranean clade, new viral variants of WMV and CMV were found. Concretely, 7 new WMV profiles and a reassorting CMV isolate (IB-IB-IA) were observed. Moreover, the complete genome of the newly described WMV isolates was sequenced. Further studies should be done to determine if these new variants spread to new areas and if they can overcome the previously described resistances.

Keywords: cucurbits, epidemiology, WMV, CMV, phylogenetics, recombination

1. Introduction

The cultivation of cucurbits is of major economic importance in Spain, which is the principal exporter of these crops in Europe, with an annual production of more than 3.5 million tonnes. Melon (*Cucumis melo*), watermelon (*Citrullus lanatus*), zucchini (*Cucurbita pepo*) and pumpkin (*Cucurbita maxima* and *C. moschata*) are widely cultivated in the Spanish Mediterranean area. Unfortunately, the good climatic conditions that allow Spain to be considered the vegetable garden of Europe, also favor the spread of different pests and diseases. The incidence of viral infections might threat the growth of cucurbits in this region, affecting yield as well as the organoleptic quality of the collected fruits. To date, 59 different viruses have been described infecting curcurbits and at least 28 of them have been reported in the Mediterranean basin (Lecoq & Desbiez, 2012). The potyviruses watermelon mosaic virus (WMV) and zucchini yellow mosaic virus (ZYMV), the polerovirus cucurbit aphid-borne yellows virus (CABYV) and the cucumovirus cucumber mosaic virus (CMV), which are aphid-transmitted, are the most important viruses in open fields, since they have been detected in more than half of the Mediterranean countries, causing major economic losses (Lecoq & Desbiez, 2012). Additionally, Moroccan watermelon mosaic virus (MWMV) was first described affecting zucchini crops in Europe more than twenty years ago, but it has not spread as much as other potyviruses (De Moya-Ruiz *et al.,* 2021; Desbiez *et al.*, 2020).

Some whitefly-transmitted viruses, such as the crinivirus beet pseudo-yellows virus (BPYV) have been present in the Mediterranean basin for many years but the emergence of the crinivirus cucurbits yellow stunting disorder virus (CYSDV) in the late 1990s, rapidly replaced BPYV in some areas. CYSDV causes major economic losses, and it is considered one of the most damaging recent emerging viruses in cucurbits (Lecoq & Desbiez, 2012). In 2011 the crinivirus cucurbit chlorotic yellows virus (CCYV) was first reported in Europe, concretely in Greece, causing symptoms

similar to those produced by CYSDV (Orfanidou *et al.,* 2014). Even though CCYV has spread to other Mediterranean countries (Maachi et al., 2022), it has not been yet as problematic in open field conditions as it is in the United States, where both CCYV and CYSDV have been detected in a high percentage of samples tested on both the East and West coasts (Kavalappara *et al.*, 2021; Mondal *et al.*, 2023). Another whitefly-transmitted virus that causes severe economic losses in greenhouse and open-field cucurbits crops is the tomato leaf curl New Delhi virus (ToLCNDV), that was first reported in Europe in 2012 in Spain (Juárez *et al.*, 2014) and is rapidly spreading. Contact- and seed-transmitted viruses from the genus *Tobamovirus* are also emerging worldwide. Among them, cucumber green mottle mosaic virus (CGMMV) is the most economically important and it has achieved a global distribution. This virus has been present in Southern Spain since the 1990s and lately different outbreaks have taken place in greenhouses located in that area (Crespo *et al.*, 2017).

The already complex epidemiological situation can be complicated even more as a consequence of the appearance of new viral strains that may arise from recombination events, or introduced by international trade, since they might cause more severe symptoms and reduce the efficiency of genetic resistances (Desbiez et al., 2021). This is the case of WMV, in which the `emergent´ groups (EM) that were first reported in France in the year 2000 have completely displaced the `classic´ strains that used to be predominant in the Mediterranean region (Bertin et al., 2020; Desbiez *et al*., 2020). These `emergent´ isolates have been connected with more severe symptoms observed since that year in open-field conditions. Moreover, new genetic variants have been found within the WMV groups (Desbiez et al., 2020), and some of them seem to overcome the genetic resistances previously described (Desbiez *et al.*, 2021). New recombinant strains of CABYV causing more severe yellowing symptoms have recently been detected (Rabadán *et al.*, 2021), and they have had an important economic impact on the production of

cucurbits. Also, introduction of new strains of CMV (Jacquemond, 2012), CGMMV (Crespo *et al.*, 2017) and ZYMV (Lecoq & Desbiez, 2012) with Asian origin have been reported during the last decades.

Conducting virus incidence surveys to monitor introduction/spread of viruses in the main cucurbits producing areas is essential to detect the main viral infections and to study the genetic variability of the viruses. This allows the mitigation of threats for agriculture, by designing the most effective methods to manage these viruses and their vectors.

Historically, CMV, WMV and CABYV have been the most prevalent viruses in Spain, affecting all the main cucurbits crops and producing areas (Kassem *et al.*, 2007; Juárez *et al.*, 2013). ZYMV has also been detected in previous studies, but at lower infection rates (Kassem *et al.*, 2007; Juárez *et al.*, 2013) whereas CYSDV caused important economic losses during the late 1990s but its incidence decreased in the early 2000s (Juárez *et al.*, 2013). Recent surveys carried out on watermelon and pumpkin fields in 2018-2020 summer seasons showed that WMV and CABYV were still the most common viruses found in plants from both crops, with mixed infections being common (De Moya-Ruiz *et al.*, 2021; Rabadán *et al.*, 2021, 2023). MWMV, CMV and ZYMV were also found in these surveys but at much lower rates. Nevertheless, these surveys did not analyze the presence of whitefly-transmitted viruses such as CYSDV, ToLCNDV, or CCYV, the latter detected for the first time in Spain in 2018 (Chynoweth *et al.*, 2021). Neither was the seed- and contact-transmitted virus CGMMV.

The aim of this study was to increase the knowledge of the current status of viral infections in melon, watermelon, zucchini and pumpkin crops in Spain. The occurrence of 9 different viruses in the main cucurbits producing areas of the Spanish Mediterranean region was monitored. Additionally, the molecular diversity of these viruses in the different regions was further studied.

2. Materials and methods

2.1 Sample collection

Surveys in conventional farming fields were carried out during early and late July in 2019 and 2020, corresponding to the middle and end of the main growing season of cucurbits crops in Eastern Spain. In 2020 surveys were also carried out in September, as it is the harvesting time of squashes and pumpkins. In 2019 a total of 10 locations were monitored in 6 provinces (Valencia, Castellón, Málaga, Almería, Murcia and Ciudad Real) belonging to 4 regions (Comunidad Valenciana, Andalucía, Región de Murcia and Castilla la Mancha) and during 2020 samples were collected in 5 locations distributed in 4 provinces (Valencia, Málaga, Ciudad Real and Badajoz) of 4 Spanish regions (Comunidad Valenciana, Andalucía, Castilla la Mancha and Extremadura) (**Table S1**). During 2019 and 2020, a total of 170 and 132 apical leaf samples, respectively, with symptoms of virus infection, such as mosaic, yellowing or foliar deformation, were collected from melon, zucchini and pumpkin grown in open fields. Moreover, 323 apical leaves of symptomatic plants were also collected in an organic melon field (1 plot) located in La Punta (province of Valencia in Comunidad Valenciana) in 2019, to explore whether there might be different results depending on the farming system. Additionally, plots located in Museros and La Punta (province of Valencia in Comunidad Valenciana) were monitored, respectively, at 3 and 4 different dates during summer season to evaluate the viral incidence changes over time.

2.2 Virus detection

To detect all the studied RNA viruses (WMV, MWMV, CMV, CYSDV, ZYMV, CGMMV, CCYV and CABYV) RT-PCRs were carried out. Total RNA was extracted from young leaves using Extrazol ® EM30 (BLIRT S.A.) following the manufacturer´s instructions. RNA concentration was measured using spectrophotometry in a NanoDrop ND-1000 spectrophotometer v.3.5. and 1 µg of RNA was reverse-transcribed with the

RevertAid™ RT First Strand cDNA Synthesis Kit (ThermoFisher Scientific) using random primer and following the manufacturer´s recommendations. The obtained cDNA was directly used for the PCR amplification of the CP gene -regions of WMV, MWMV, CMV, CYSDV, ZYMV, CCGMV, CCYV and CABYV (**Table S2**). Moreover, to make the results comparable with previous studies carried out in France, the primers described by Desbiez *et al.* (2020) to sequence different regions of WMV, CMV and CABYV genomes were also used (**Table S2**) for both PCR amplification and sequencing (see section below). All the PCRs were carried out with the DreamTaq Green PCR Master Mix (ThermoFisher Scientific) following the same cycling conditions: 94°C 5min, (94°C 30s, 55°C 30s, 72°C 45s) x32 cycles, 72°C 5min, 10°C. We used 1 µl of template, 1X DreamTaq Green PCR Master Mix, 0.1 µM of each primer, 0.14 mM of dNTPs and 0.01U/µl of Taq DNA polymerase in a final volume of 30 µl. Two independent replicates were performed for each cDNA sample to reduce the risk of false negatives. In order to detect ToLCNDV, the only DNA virus screened during the surveys, a tissue printing assay was performed as described by Sáez *et al*. (2021). Total DNA was also extracted from samples from conventional farming fields where ToLCNDV had been detected in the tissue-printing analysis and from some random symptomatic samples from the organic farming field in which ToLCNDV had also been detected. Total DNA extraction was carried out using the CTAB method with minor modifications (Sáez *et al.*, 2021) . DNA concentration was measured using spectrophotometry in a NanoDrop ND-1000 spectrophotometer v.3.5. and the DNA was diluted to 50 ng/µl. PCR amplification of the DNA-A coat protein gene of ToLCNDV was done with the same primers used to synthesize the corresponding probe (**Table S2**) and following the conditions previously indicated.

2.3 Sequencing of viruses in cucurbits

For all the viruses found to be present in the surveys, one positive sample of every crop tested positive in each location and plot at different dates was chosen, and the corresponding PCR product was purified using the EXTRACTME DNA CLEAN-UP KIT (BLIRT S.A.) and paired-end sequenced by Sanger method (Secuenciación de ADN y análisis de la expression génica, Instituto de Biología Molecular y Celular de Plantas (IBMCP), Valencia, Spain) (**Table S2, S3, S4, S5, S6 and S7**). Moreover, in the case of WMV, one isolate of each of the different detected profiles (see phylogenetic analysis) was chosen to be fully sequenced. The full-length WMV genome was paired-end sequenced by Sanger, using a set of 19 internal primers (**Table S4, S8**) that produced overlapping sequences of 300-900 bp. The PCRs were conducted as previously described. The quality of the obtained fragment sequences was manually checked using Chromas (Technelysium DNA Sequencing Software) and the consensus sequence of the complete genome of the WMV isolates was obtained using CAP3 sequence assembly program (www.doua.prabi.fr/software/cap3).

2.4 Sequence analyses

The obtained sequences of the different viruses were aligned using MUSCLE, included in MEGA11 (Tamura *et al.*, 2021). Worldwide reference sequences of the same viruses were retrieved from GenBank (www.ncbi.nlm.nih.gov/genbank/) and included in the alignments and phylogenetic analysis. MODELTEST, included in MEGA11, was used to calculate the best model of multiple substitutions. Maximum-likelihood trees were constructed with MEGA11, using the previously selected substitution model and 500 bootstrap replicates. Clusters were defined based on the structure of the constructed trees. `Profiles´ were defined as the combination of groups for the different genetic fragments, as described in Desbiez *et al.* (2020).

Moreover, the detection of potential recombination sites within the complete WMV sequences was performed with RDP4 (Martin *et al.*, 2015). The program was used with the default settings and a Bonferroni corrected *p-value* ≤ 0.01. Different analytical methods were used: RDP, GENECONV, Chimaera, MaxChi, BOOTSCAN, 3Seq and SISCAN. Only those recombination events detected by four or more methods were considered as significant. For WMV isolates, pairwise identities of nucleotide and translated amino acid sequences were determined using MEGA11 (Tamura *et al.*, 2021). Furthermore, DNAsp6 (Rozas *et al.*, 2017) was used to calculate the dN/dS ratios (ω), which compares the synonymous substitution rates (dS) – assumed to be neutral - with nonsynonymous substitution rates (dN), which are exposed to selection as they change the amino acid composition of a protein.

3. Results

3.1. Virus occurrence in Comunidad Valenciana

Among the 121 symptomatic samples collected under conventional farming conditions in Comunidad Valenciana during the summer season of 2019, 76 of them (62.8%) were infected by, at least, one of the viruses identified in this area (WMV, MWMV, CMV, CABYV) (**Table 1, Figures 1a S1a**). The tested viruses were most common in symptomatic squash plants compared to symptomatic plants of other crops with 68.5% of infected plants, followed by watermelon (61.9%) and zucchini (57.1%), whereas only 50.0% of the melon samples were diagnosed as infected (**Table 1**, **Figure 1a**). In 2020, 115 of the 121 tested samples (95.0%) were infected (**Table 1, Figure 1b**) (**Figure S1b**). The percentage of infected melon and squash samples was similar (99.2% and 95.4%, respectively), while it was lower among watermelons (85.7%) (**Table 1**).

Table 1. Proportion of samples infected by the studied viruses in 2019 and 2020 in all the monitored areas under conventional farming conditions.

Year	Region	Host	No. positive/ No. samples	WMV	CMV	CABYV	ToLCNDV	MWMV
2019	C. Valenciana	Melon	9/18 (50.0%)	7	2	2	0	1
		Watermelon	26/42 (61.9%)	19	14	8	0	0
		Squash	37/54 (68.5%)	34	8	11	0	2
		Zucchini	4/7 (57.1%)	0	2	3	0	0
		Total	76/121 (62.8%)	60	26	24	0	3
	Andalucía	Melon	17/19 (89.5%)	12	13	13	1	0
		Watermelon	2/4 (50.0%)	2	0	2	0	0
		Squash	1/1 (100%)	1	0	1	0	0
		Zucchini	2/2 (100%)	2	2	2	0	0
		Total	22/26 (84.6%)	17	15	18	1	0
	Murcia	Melon	11/12 (91.7%)	3	2	8	1	0
		Watermelon	2/8 (25.0%)	1	1	0	0	0
		Total	13/20 (65.0%)	4	3	8	1	0
	Castilla la Mancha	Melon	3/3 (100%)	3	2	3	2	0
		Total	3/3 (100%)	3	2	3	2	0
2020	C. Valenciana	Melon	41/42 (99.2%)	41	28	29	0	0
		Watermelon	12/14 (85.7%)	10	9	6	0	0
		Squash	62/65 (95.4%)	52	47	42	0	1
		Total	115/121 (95.0%)	103	84	77	0	1
	Andalucía	Melon	5/6 (83.3%)	5	2	2	0	0
		Zucchini	2/2 (100%)	2	1	0	0	0
		Total	7/8 (97.9%)	7	3	2	0	0
	Extremadura	Melon	2/2 (100%)	2	0	1	0	0
		Watermelon	1/1 (100%)	0	0	1	0	0
		Total	3/3 (100%)	2	0	2	0	0
	Castilla la Mancha	Melon	3/3 (100%)	0	3	3	0	0
		Total	3/3 (100%)	0	3	3	0	0

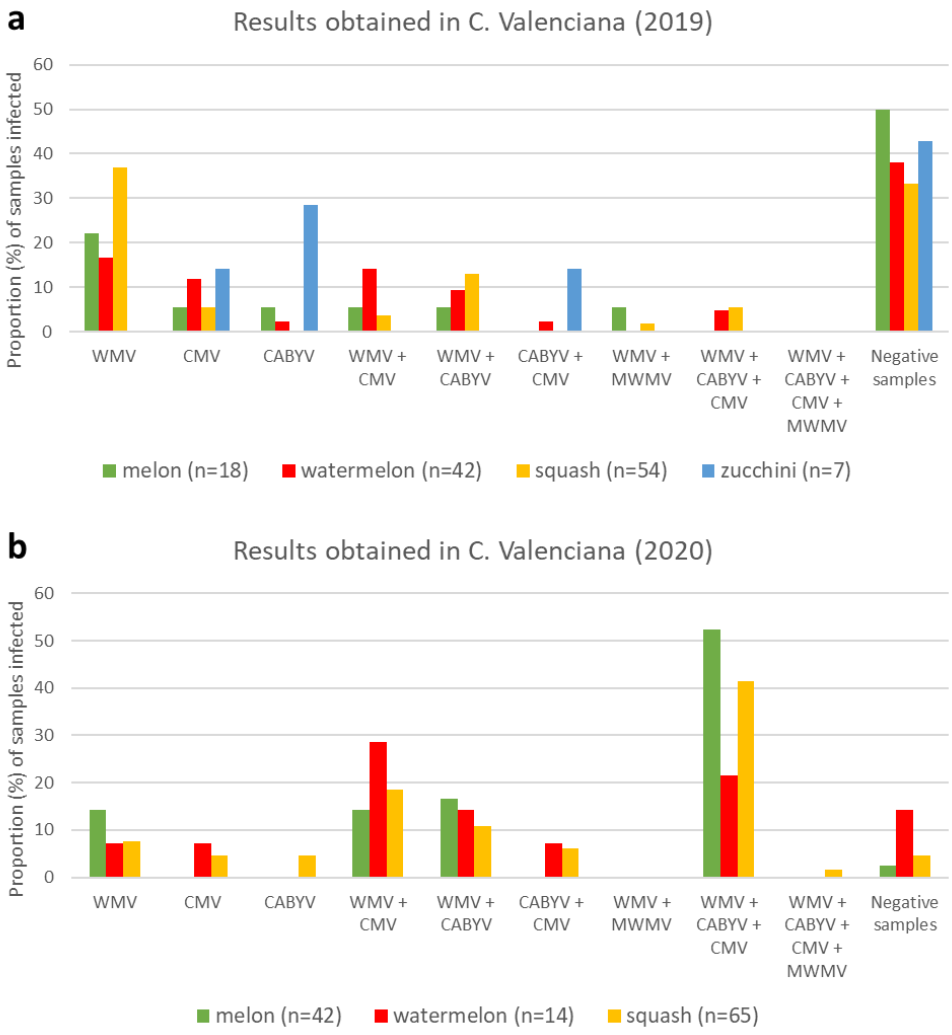

Figure 1. Detailed results obtained in Comunidad Valenciana during 2019 (a) and 2020 (b), indicating the proportion of single and mixed infections observed per crop. The number of samples tested is indicated for each crop.

WMV was the most prevalent virus, followed by CMV and CABYV. Only 3 and 1 samples were diagnosed as infected by MWMV in 2019 and 2020 respectively, while ZYMV, CGMMV, ToLCNDV, CYSDV and CCYV were not detected in the monitored areas (**Table 1**; **Figure 1**). Mixed infections were common, representing 24.8% and 79.3% of the total samples in 2019 and 2020, respectively. Mixed

infections of WMV and CABYV or WMV and CMV were frequently observed (**Figure 1**). Triple infection by WMV, CABYV and CMV represented 4.1% of cases during 2019, while in 2020 it increased to 79.3%. Only one squash sample was diagnosed with the four detected viruses (WMV, CABYV, CMV and MWMV) (**Figure 1**). When the temporal line was studied at the locality of Museros (Province of Valencia in Comunidad Valenciana), it was noticed that single infections were more common during mid-July and the proportion of mixed infections increased over time (**Figure S2**). WMV was the most prevalent virus in all the studied crops in early and mid-July, maintaining its relative importance over time, while CABYV, CMV and MWMV mostly appeared in mixed infections with WMV in late July (**Figure S2**).

3.2. Virus occurrence in other areas

In order to determine the genetic variability of the detected viruses in different geographical regions, surveys were also carried out in other major producing areas. Hence, during 2019, 20, 26 and 3 symptomatic cucurbit samples were collected from open fields in Murcia, Andalucía and Castilla la Mancha, respectively. In these areas CABYV was the most prevalent virus, followed by WMV and CMV. No samples were infected by MWMV, ZYMV, CGMMV, CYSDV or CCYV, while one melon sample in Andalucía, one in Murcia and two melons from Castilla la Mancha were diagnosed as infected by ToLCNDV (**Table 1**). Mixed infections were also common in these areas. During 2020, 8, 3 and 3 symptomatic samples were collected from Andalucía, Extremadura and Castilla la Mancha respectively (**Table 1**). Once again, WMV, CMV and CABYV were the prevalent viruses, even though CMV was not detected in Extremadura and neither was WMV in Castilla la Mancha, probably due to the low number of collected samples.

3.3. Virus occurrence under organic farming conditions

A melon field located in La Punta (province of Valencia in Comunidad Valenciana) and cultivated under organic farming conditions, was monitored on four different dates during 2019 summer season. Whiteflies and aphids were frequently observed in the surveys. While acknowledging that our study was conducted solely in a single organic field, it serves as an initial exploration to assess the incidence of different viruses in organic fields compared to conventional ones. In the studied field, 98.5% of the collected symptomatic samples were diagnosed as infected by, at least, one of the tested viruses. WMV was the most prevalent virus (96.6% of the samples were infected by WMV), followed by CABYV, ToLCNDV and CMV. Only 5 samples were infected by MWMV, while CYSDV, CCYV, CGMMV and ZYMV were not detected in the survey (**Table 2**) (**Figure 2**).

Table 2. Proportion of positive samples for each of the viruses detected in the organic melon farming field at different dates.

	No. Positive/ No. samples	WMV	CABYV	ToLCNDV	CMV	MWMV
June	104/105 (99.0%)	104/105	15/105	11/105	12/105	5/105
Early July	94/94 (100%)	94/94	40/94	47/94	4/94	0/94
Mid July	16/16 (100%)	16/16	12/16	12/16	0/16	0/16
Early August	104/108 (96.3%)	99/108	69/108	44/108	27/108	0/108
Total	318/323 (98.5%)	313/323	136/323	114/323	44/323	5/323

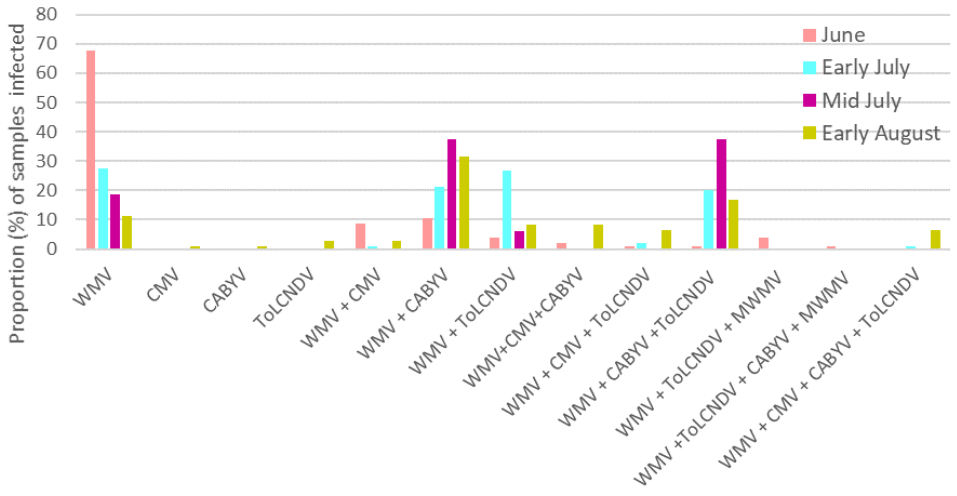

Figure 2. Temporal evolution of single and mixed infections during the summer season of 2019 in an organic melon field located in Comunidad Valenciana.

In late June most samples were infected only by WMV (67.6%) and, at later dates, the proportion of mixed infections increased. In early July, infections only by WMV were the most common (27.7%), followed by mixed infections by WMV and ToLCNDV (26.6%), WMV and CABYV (21.3%) or triple infection caused by WMV, CABYV and ToLCNDV (20.2%). In mid-July the proportion of samples infected only by WMV decreased to a 18.75% while the rate of mixed infections caused by WMV and ToLCNDV or WMV, ToLCNDV and CABYV increased to 37.5% each. In early August the lower rate of plants infected only by WMV was found (11.1%), while mixed infections by WMV and CABYV, and WMV, CABYV and ToLCNDV (31.5 and 16.7%, respectively) remained as the most predominant. At this date, CMV gained importance, as 25% of the samples were infected with this virus, appearing especially in mixed infections. Surprisingly, it was observed that percentage of plants infected by WMV, CMV, CABYV, and ToLCNDV was maintained or even increased throughout the study period. In contrast, the presence of MWMV was

only detected in the month of June. Further sampling in organic fields is warranted to facilitate a more robust comparison with conventional fields.

3.4. Molecular diversity of viruses in crops

3.4.1. Cucumber mosaic virus

To study the molecular diversity of CMV, 376, 446 and 434 or 451 bp long partial sequences of the three RNAs that comprise the genome of this virus were obtained from 23 samples that represented all the crops and locations monitored (**Table S3**). Phylogenetic analyses were performed including isolates retrieved from GenBank belonging to the three main CMV groups that have been described worldwide – namely groups IA, IB and II. None of the PCR products for viral isolates was classified within group II and 19 of the sequenced CMV isolates belonged to group IA (**Figure 3, Table S3**). Moreover, 4 samples (1 watermelon and 3 melons) collected in Museros (Valencia, Comunidad Valenciana) and Alhaurín el Grande (Málaga, Andalucía) in 2020 were infected with a reassorting isolate. For RNA1 and RNA2, these isolates clustered within group IB, whereas for RNA3, they grouped within group IA (**Figure 3, Table S3**).

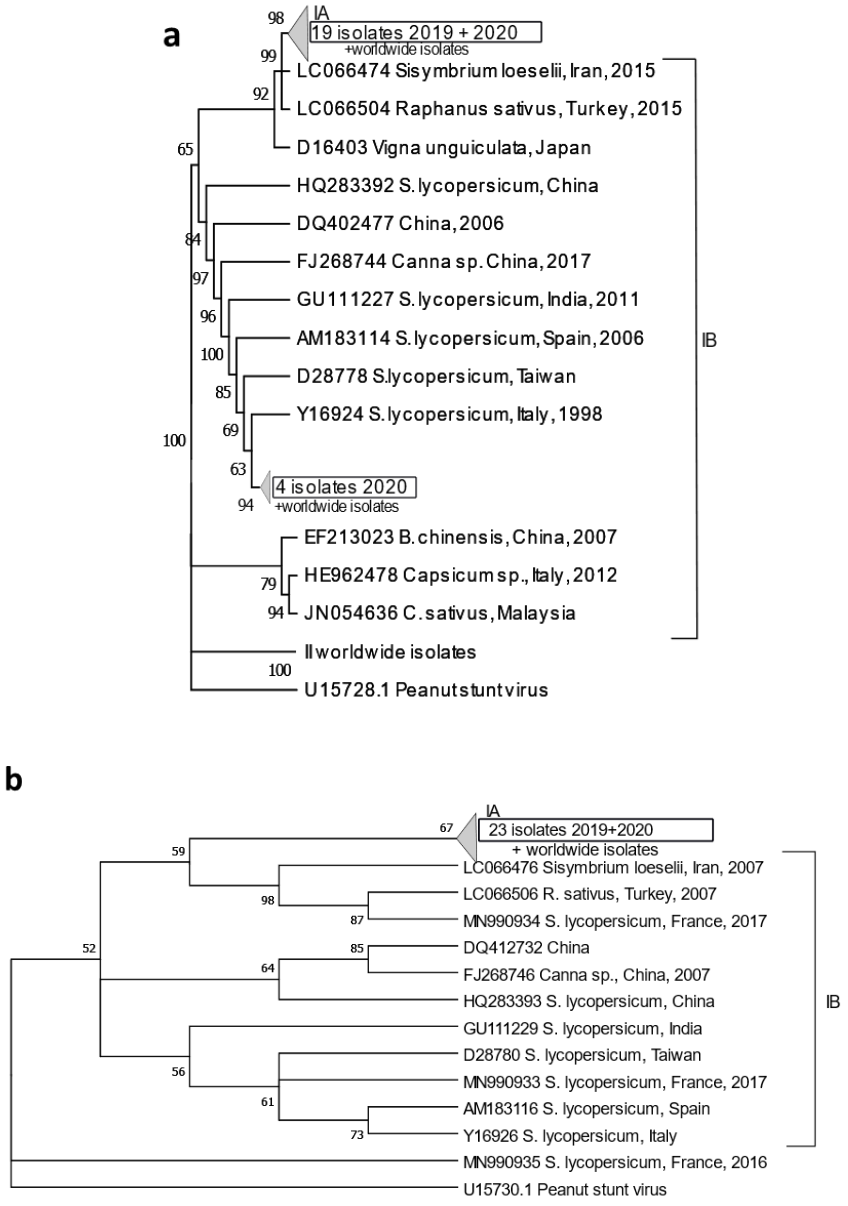

Figure 3. Maximum-likelihood trees obtained for CMV RNA1 (a) and RNA3 (b) partial sequences. Bootstrap values (n=500 bootstrap replicates) above 50% are indicated for each node. For RNA2 the same result was obtained as for RNA1. Sequences corresponding to isolates from the survey are boxed. Peanut stunt virus was used as an outgroup. The isolates sequenced in this work included within each cluster are reported in Table S3. The worldwide isolates used to construct the tree are reported in Table S9.

3.4.2. Watermelon mosaic virus

The molecular diversity of WMV was analyzed by sequencing the P3-CI (452 bp) and NIb-CP (450 or 456 bp) coding regions of 21 and 17 isolates in 2019 and 2020, respectively (**Table S4**). Phylogenetic analyses were conducted including sequences retrieved from GenBank of the `classic´ (CL) and `emerging´ (EM 1-4 and Profiles 5-11) isolates of the WMV groups that had previously been established (Desbiez *et al.,* 2020). This study revealed a high molecular diversity among the sequenced isolates, as they were classified within 9 different profiles (combinations of P3-CI and NIb-CP groups based on the phylogenetic tree structure) (**Figure 4, Table S4**). None of the partially amplified genomes were catalogued within the `classic´ group and only two isolates collected in Murcia and one isolate collected in the province of Badajoz (Extremadura) belonged to one of the 11 profiles previously described by Desbiez *et al*. (2020). Concretely, the WMV PCR products amplified from samples collected in Murcia were classified within the EM4 group and the isolate from Extremadura belonged to the Profile 10 (**Table S4**). Thereby, 7 new profiles (Profiles A-G) were established for the first time.

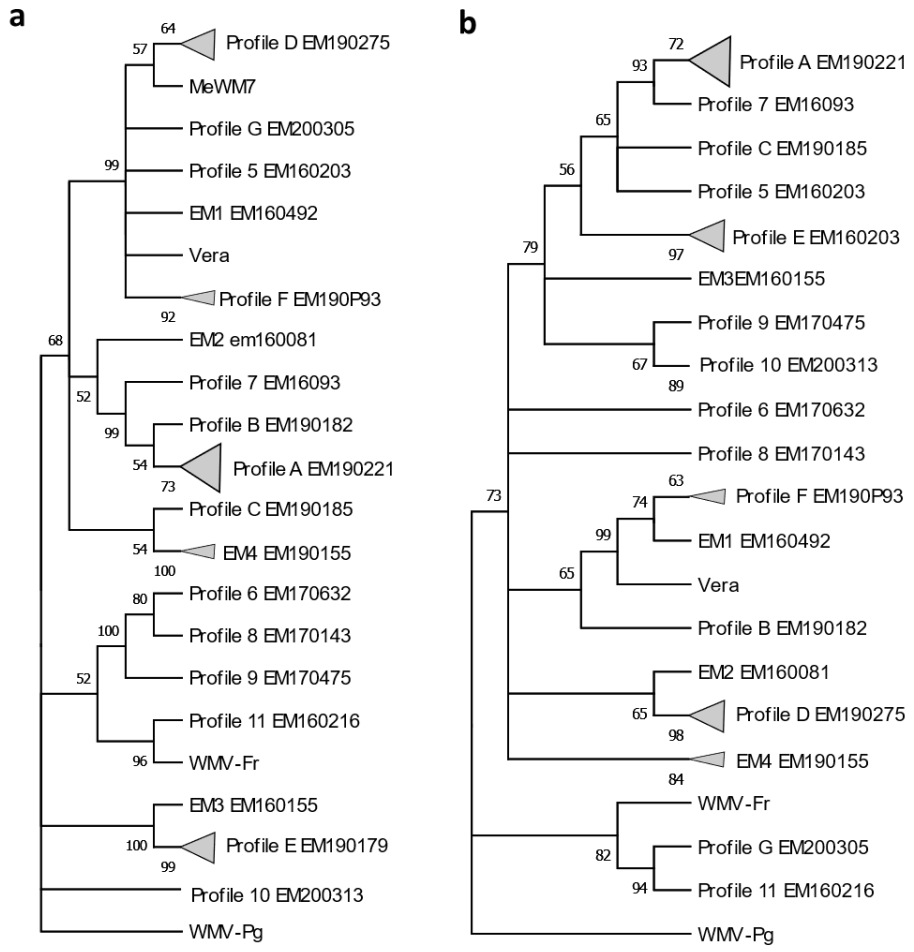

Figure 4. Maximum-likelihood trees obtained for partial nucleotide sequences of the WMV P3-CI (a) and NIb-CP (b) gene regions. Bootstrap values (n=500 bootstrap replicates) above 50% are indicated for each node. The highly divergent sequence of isolate WMV-Pg was used as an outgroup. Profile numbers (A-G) and the name of the reference isolate for each profile, as defined in Table S4, are indicated for each branch tip. The isolates included within each Profile are also indicated in Table S4.

When the geographical origin of the new profiles was studied, certain degree of co-localization of the isolates clustered within the same profile was observed (**Figure S3, Table S4**). Profiles A, C and D were only detected in Comunidad Valenciana: all the viral isolates sequenced from samples collected in Museros in 2019 grouped within profile D, while those collected in the same place but in 2020 were distributed between profiles A and D; WMV isolates obtained from samples collected in Benicarló (Castellón, Comunidad Valenciana) in 2019 were classified as profile A, while profile C was only detected within the samples collected in Gandía (Valencia, Comunidad Valenciana) in 2019. In the South, all the isolates sequenced from the province of Málaga (Andalucía), except one, were classified as profile E, and the remaining isolate, collected in 2020, was catalogued as profile G. Finally, only one isolate collected in Castilla la Mancha in 2019 was classified as profile B.

As apparent discrepancies between P3-CI and NIb-CP clustering suggested recombination events, the complete WMV genome sequence of one isolate of each profile was obtained to study the possible recombination sites, as well as the nucleotide and amino acid identity between isolates (**Table S4**). Isolates EM190179, EM190155, EM190221, EM190185, EM190182 and EM190275 were further identified as putative recombinants (Table S10). Two breakpoints within the CI coding region (4.281 -5.637 nt) were identified for the isolates EM190221 and EM190182, suggesting isolate EM190275 as the major parent and the isolate FBR04-37 (sequence EU660586) as the minor parent. On the other hand, two breakpoints were detected between HC-Pro and P3 genes (1.831-3.775 nt) in the isolates EM190185 and EM190275, suggesting the isolate FBR04-37 as the major parent and the isolate CHI87-620 (sequence EU660580) as the minor parent. Two additional breakpoints were detected in the isolate EM190179 within the P1-HC-Pro region (1137-2027 nt) suggesting isolate FBR04-37 as major parent and isolate EM190182 as the minor parent. The two last breakpoints were detected in the

isolate EM190155, within the HC-Pro coding region (1950-2344 nt), suggesting the isolate FBR04-37 as the major parent and the isolate FMF03-141 (sequence EU660583) as the minor parent. The 5´end of the HC-Pro coding region and the 3´end of the CI coding region had been described as recombination hotspots in previous studies (Desbiez *et al.*, 2020). Pairwise comparisons for the complete genome sequence of the analyzed profiles were carried out (**Figure 5a, 5b**) (**Figure S4**). Sequences from the isolates FMF00-LL1 (group EM1), FMF03-141 (group EM2), FBR04-37 (group EM3), C05-270 (group EM4) and WMV-Fr (`classic´ group) (sequences EU660581, EU660583, EU660586, EU660585 and AY437609, respectively) and of the WMV Spanish isolates most recently described, Vera and MeWM7 (sequences MH469650 and MW147356, respectively), were also included in the analysis. The nucleotide sequence identity ranged from 89.6% to 99.5%, whereas the amino acidic sequence identity was between 94.1% and 99.7% (**Figure S4**), which is consistent with the observed low dN/dS ratios (ω), which compares the synonymous substitution rates (dS) – assumed to be neutral - with nonsynonymous substitution rates (dN), which are exposed to selection as they change the amino acid composition of a protein (**Figure 5c**). The highest identity, at both nucleotide and amino acid levels, was found between some of the isolates found to be infecting samples collected in Comunidad Valenciana (Profiles C, D and F) and in Andalucía (Profile G), and these sequences were also highly similar to Vera and MeWM7 isolates, and also to the isolate FMF00-LL1, which belonged to the EM1 group (**Figure 5a, 5b**) (**Figure S4**). Isolates of Profile B, which were found infecting samples collected in Castilla la Mancha, also showed similar identity ratios. Considering isolates of Profile A, which were detected in plants collected in both Benicarló and Museros, a lower identity level was observed with the previously named isolates (Profiles C, D, F, G and B) (**Figure 5a, 5b**) (**Figure S4**). On the other hand, the genome sequence of Profile E, collected in Málaga, and Profile 10, collected in Badajoz, showed a high similarity between them and with EM3

sequences (**Figure 5a, 5b**). Isolates of EM4 and Profile 10 were the most divergent at both nucleotide and amino acidic levels, showing, on average, higher non-synonymous substitutions rates (**Figure 5c**) (**Figure S4**). However, it should be considered that these analyses have been conducted using only one isolate per group or profile. Hence, slight differences could be obtained when analyzing a greater variability within each cluster. In any case, these results constitute an initial approximation to better understand the variability between profiles.

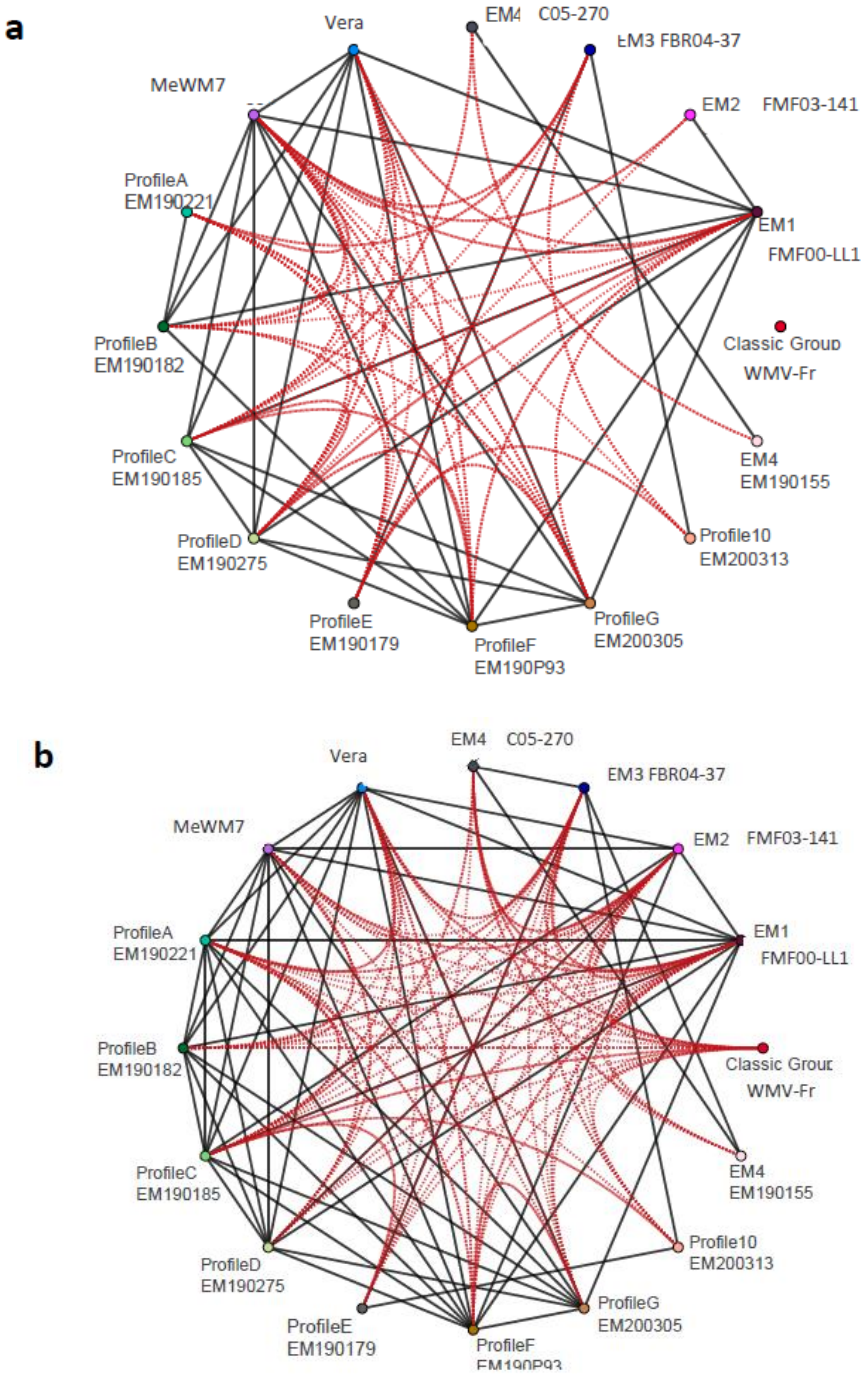
a
Vera
EM4 C05-270
EM3 FBR04-37
MeWM7
EM2 FMF03-141
ProfileA
EM190221
EM1
FMF00-LL1
ProfileB
EM190182
Classic Group
WMV-Fr
ProfileC
EM190185
EM4
EM190155
ProfileD
EM190275
Profile10
EM200313
ProfileE
EM190179
ProfileF
EM190P93
ProfileG
EM200305
b
Vera
EM4 C05-270
EM3 FBR04-37
MeWM7
EM2 FMF03-141
ProfileA
EM190221
EM1
FMF00-LL1
ProfileB
EM190182
Classic Group
WMV-Fr
ProfileC
EM190185
EM4
EM190155
ProfileD
EM190275
Profile10
EM200313
ProfileE
EM190179
ProfileF
ProfileG
EM200305

Figure 5.

c

Location			C. Valenciana	Castilla la Mancha	C. Valenciana	C. Valenciana	Andalucía	C. Valenciana	Andalucía	Extremadura	Región de Murcia	C. Valenciana (2016)	Región de Murcia (2019)	France (2000)	France (2003)	France (2004)	France (2005)
	Profile		Profile A	Profile B	Profile C	Profile D	Profile E	Profile F	Profile G	Profile 10	EM4			EM1	EM2	EM3	EM4
		Isolate	EM190221	EM190182	EM190185	EM190275	EM190179	EM190P93	EM200305	EM200313	EM190155	Vera	MeWM7	FMF00-LL1	FMF03-141	FBR04-37	C05-270
C. Valenciana	Profile A	EM190221															
Castilla la Mancha	Profile B	EM190182	0.06														
C. Valenciana	Profile C	EM190185	0.04	0.04													
C. Valenciana	Profile D	EM190275	0.05	0.04	0.03												
Andalucía	Profile E	EM190179	0.06	0.05	0.05	0.05											
C. Valenciana	Profile F	EM190P93	0.04	0.04	0.02	0.04	0.05										
Andalucía	Profile G	EM200305	0.04	0.04	0.03	0.05	0.05	0.04									
Extremadura	Profile 10	EM200313	0.05	0.05	0.05	0.05	0.05	0.05	0.05								
Región de Murcia	EM4	EM190155	0.05	0.05	0.06	0.06	0.04	0.06	0.06	0.04							
C. Valenciana (2016)		Vera	0.04	0.04	0.02	0.04	0.05	0.04	0.04	0.05	0,057						
Región de Murcia (2019)		MeWM7	0.05	0.04	0.03	0.1	0.05	0.04	0.05	0.05	0.06	0.04					
France (2000)	EM1	FMF00-LL1	0.05	0.04	0.03	0.05	0.05	0,070	0.04	0.05	0.06	0.09	0.05				
France (2003)	EM2	FMF03-141	0.05	0.05	0.05	0.06	0.05	0.05	0.05	0.05	0.05	0.06	0.06	0.06			
France (2004)	EM3	FBR04-37	0.05	0.05	0.04	0.05	0.05	0.05	0.04	0.05	0.04	0.04	0.05	0.05	0.04		
France (2005)	EM4	C05-270	0.05	0.05	0.06	0.06	0.04	0.06	0.06	0.04	0.06	0.06	0.06	0.06	0.06	0.03	
France	Classic Group	WMV-Fr	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.04	0.05

Figure 5. Continuation. Pairwise identities found for the different WMV profiles sequenced, reference isolates were also included in the analysis. (a) Proportion (%) of identity between nucleotide sequences: black lines indicate a global identity >95% and red lines show a local 100% identity between regions >500bp. (b) Proportion (%) of identity between amino acid sequences: black lines indicate a global identity >97% and red lines show a local 100% identity between local sequence regions >500 amino acids. (c) Pairwise comparisons of the dN/dS ratio (non-synonymous substitution rates / synonymous substitution rates). The origin of the reference isolate, as well as the profile within they were clustered is indicated. EM 1-4: emerging groups 1-4.

The position in which the amino acid changes were taking place in the sequences was further analyzed. It was observed that most of the amino acid sequence differences detected between the sequenced isolates were located within the P1, HC-Pro and P3 gene regions and a lower number of differences were also detected at the CI and CP gene regions (**Figure S5**). This accumulation of sequence differences within the P1 sequences had already been observed when the complete sequence of the Spanish isolates MeWM7 and Vera had been studied (De Moya-Ruiz *et al.*, 2021). The dN/dS ratio was then studied separately for each gene of the WMV genome. No signs of positive selection were observed in any of the genes. However, the ratios obtained for the P1 gene were the highest (ranging from 0.11 to 0.60). When the amino acid sequences were compared, it was observed that most of the differences not previously described in the newly sequenced Spanish isolates (Vera and MeWM7) or in `classic´ isolates (WMV-Fr) were already present in the sequences of the `emerging´ isolates (groups EM1-EM4) previously detected in France (FMF00-LL1, FBR04-37, C05-270 and FMF03-141). However, several amino acid sequence differences with respect to the Spanish, the `classic´ and `emerging´ isolates were detected for the first time (**Table 3**). Furthermore, based on the BLOSUM 62 matrix scores, a significant number of these recently identified amino acid modifications were categorized as non-conservative. The BLOSUM matrix scores quantify the likelihood (log-odds) of one amino acid being replaced by another within a collection of protein sequence alignments.

Table 3. New amino acid sequence differences within each protein from the complete genomes of the different WMV profiles sequenced.

	Profile A[a] (EM190221)	Profile B (EM190182)	Profile C (EM190185)	Profile D (EM190275)	Profile E (EM190179)	Profile F (EM190P93)	Profile G (EM200305)	EM4 (EM190155)	Profile 10 (EM200313)
P1	V113I[b], H131Q*, E135K, K261L***, S278F***, K298R, D307N, T331M**, K386R, S410A, E437K	N274S, T275A, T279I**, F285L, S292L***, I438V		R286K, V349I, H364Y	V18A, P179S**, A153I, V154A, K157_del[c]***, S179N, I216V, N274D, A320V	A203V	K336R	H119Y	E93K, A153I**, V154A, S171F***, I216V, K241E, K261E, V302I, K361R, A387P**
HC-Pro	Q226K	T77I**		T398I**					L76A**, R90Q, k100R
P3	K217R, N293M***	I81V, A207V, N293M***	V149L, K281R, P332S**		F223I*	A230V	N133H		M36L, K217R, E317D, T338A
6K1	L19M	L19M							
CI	N484S, T557I**	N484G, T557I**	D225E, G249_del[c]***, R250_del[c]***, P486S**		I8M, S23N, R366K, L502_A517_del***			P361S**	I49V, I69T, G239S, K446N*, I471L

	Profile A[a] (EM190221)	Profile B (EM190182)	Profile C (EM190185)	Profile D (EM190275)	Profile E (EM190179)	Profile F (EM190P93)	Profile G (EM200305)	EM4 (EM190155)	Profile 10 (EM200313)
6K2									
NIa-VPg					K177R			Y119H	R90K
NIa-Pro									
NIb	M400I	V31I, M400T**		M400I		V94I	S145G*, K279R		R359K
CP		Q92P**, K113R, V114T*		G36D**, Q92P**, V114T*, M279K**	N30S	P28A**	G2K***, K3Q, D16E, G23D**, D26N, T33V*,		

[a]: Amino acid sequence differences represented are between each of the profiles sequenced in his work and reference isolates from the classic (sequence accession number AY437609) and emerging groups (EM1-4) (sequence accession numbers EU660581, EU660583, EU660586, EU660585), as well as in other Spanish isolates previously sequenced (sequence accession number MH469650 and MW147356). Only changes different from those previously described in the reference isolates used are presented.

[b]: The sequence difference is expressed including the most frequent amino acid previously described for each of the positions; i.e., in the change V113I for PI, the most frequent amino acid in the reference isolates used in the comparison was V, and I was first reported in the isolates sequenced.

[c]: del: deletion of one or several amino acids.

*: amino acid changes punctuated as 0 as a function of the BLOSUM 62 matrix (Negative BLOSUM scores correspond to amino acid pairs with a low likelihood of substitution, while a positive score denotes commonly observed amino acid pairs)

**: amino acid changes punctuated as -1 as a function of the BLOSUM 62 matrix.

***: amino acid changes punctuated as -2 as a function of the BLOSUM 62 matrix.

3.4.3. Cucurbit aphid-borne yellows virus

Partial sequences of CABYV CP (423 bp) and RdRp (419 bp) genes were obtained for 17 samples in 2019 and 10 samples in 2020 (**Table S5**). The variability for the sequenced region of the RdRp gene (maximum sequence divergence: 9.3%) was much higher than for the sequenced region of the CP gene (up to 4.0% divergence). Compared to the worldwide diversity, the phylogenetic analysis showed that all the partially sequenced isolates belonged to the `European-African´ clade and they were highly similar to isolates previously described in Spain (Rabadán *et al.*, 2021) and France (Desbiez *et al.*, 2020). Four molecular clusters were defined for the sequenced region of the RdPp gene and two clusters for the sequenced region of the CP gene, but with a low bootstrap support (data not shown). The same problem had been observed in previous works (Desbiez *et al.*, 2020).

3.4.4. Other viruses

The other two viruses observed in the survey, MWMV and ToLCNDV, showed a low prevalence and partial CP gene sequences (638 and 505 bp, respectively) were obtained for 4 and 5 isolates, respectively (**Table S6, S7**). Regarding the 4 MWMV isolates, the identity between the sequences was approximately 99% and they shared >98.0% of identity with isolates previously detected in Spain (ZuM10 and ESP) (sequences MW161172 and EF579944, respectively). Consequently, they were clustered within the `Mediterranean´ clade in the phylogenetic tree (**Figure S6a**). Regarding the ToLCNDV isolates, all of them clustered within the `ToLCNDV-ES´ clade (**Figure S6b**). The isolates that belong to the ToLCNDV-ES strain have been present in the Mediterranean area since it was detected in Spain in 2012 (Juárez *et al.*, 2014). The sequence identity between the isolates was >99.5% and it was also >99.5% when comparing the studied sequences with the most recently described

Spanish isolates ES-Alm-Mel-16, ES-Alm-Zuc-16 and ES-Alm-Cuc-16 (sequences LC596381, LC596382 and LC596380, respectively).

4. Discussion

In order to characterize the principal viruses affecting cucurbits crops in Spain, extensive surveys in Southeastern regions and other important areas for cultivation of cucurbits were conducted in 2019 and 2020. The data presented here showed that aphid-transmitted viruses were more established than those transmitted by whiteflies and that the newly discovered viruses had not yet spread. Concretely, WMV and CABYV were the most prevalent viruses in all the crops and monitored areas regardless of the farming system. Both viruses were found in single and mixed infections, with WMV appearing in single infections earlier in the growing season. These results were consistent with those observed in melon and squash fields in the same regions in 2003-2006 (Kassem *et al.*, 2007; Juárez *et al.*, 2013) and also in melon, watermelon and squash fields monitored in other areas in 2018-2020 (De Moya-Ruiz *et al.*, 2021; Rabadán *et al.*, 2021; Maachi *et al.*, 2022). This same trend was also observed in other Mediterranean countries (Desbiez et al. 2020). Nevertheless, several differences concerning the other studied viruses were observed. It was remarkable that, even though, CMV was present only at a low incidence or was not even detected in other surveys (De Moya-Ruiz *et al.*, 2021), it was the third most important virus in our surveys, affecting a 26.0% and 66.6% of the tested symptomatic plants grown in conventional farming fields in 2019 and 2020, respectively. Regarding the studied organic farming field, 13.6% of the symptomatic plants were infected with CMV in 2019. This situation contrasted with the results obtained in relation to ZYMV and MWMV, the other two viruses transmitted by aphids studied. On the one hand, ZYMV was not detected in this work, which is consistent with the low infection rates observed in previous studies (De Moya-Ruiz *et al.*, 2021). On the other hand, MWMV was detected in a low

number of samples in both organic and conventional farming fields. This virus was detected in Spain for the first time in 2012, but it has not become established till date. This trend had also been observed in other Spanish areas and different Mediterranean countries (Desbiez *et al.*, 2020; De Moya-Ruiz *et al.*, 2021). It seems that mixed infections might be the key to explain the different infection rates observed. It has been proven that the combination of a potyvirus and a non-related virus has a synergistic effect on the non-related virus, as it can take advantage of the RNA silencing suppression function of HC-Pro of the potyvirus (Valli *et al*., 2018). In mixed infections with CMV-WMV and CMV-ZYMV the viral load of the cucumovirus and its long-distance movement in the plant were shown to increase (Mochizuki *et al*., 2016). In contrast, the combination of two potyviruses seems to be neutral or beneficial for one virus with an antagonistic effect on the other virus. In mixed infections between ZYMV and WMV the latter virus accumulated to significantly lower levels (Salvaudon *et al*., 2013). However, WMV could benefit from the increased vector traffic for its own transmission (Salvaudon et al., 2013). This differed from mixed infections with WMV and MWMV in zucchini, where the titer titer of WMV appeared to be steady while MWMV was antagonized (De Moya-Ruiz *et al.*, 2021). Moreover, MWMV is principally transmitted by *Myzus persicae* and to a lesser extent by *Aphis gossypii*, which is more likely to be found in cucurbits fields. These data, in addition to the fact that MWMV is not able to overwinter in weeds (Desbiez *et al.*, 2020), would explain the low incidence of this potyvirus.

CGMMV was not detected in the monitored fields. The absence of this virus could be explained by two factors: its narrow host range is limited to cucurbits, and the fact that it is not transmitted by vectors, such as aphids or whiteflies. CGMMV is a seed, soil, and mechanical-transmitted virus, which explains its higher incidence in crops grown under greenhouse conditions, where pruning and other cultural practices facilitate its propagation.

Regarding whitefly-transmitted viruses, their situation in cucurbits fields had not recently been studied in Southeastern Spain. It has been noticed that the crinivirus CYSDV, which was prevalent in 2003-2004 in Southern Spain (Kassem *et al.*, 2007), was not longer detected. This contrasts with recent results obtained in Greece and Cyprus, where high infection rates were observed in both open field and greenhouses (Orfanidou *et al.*, 2019). The emerging virus CCYV, that was detected in three independent cucumber green houses in Southern Spain for the first time in 2018 and in one melon field in Murcia in 2020 (Chynoweth *et al.*, 2021; Maachi *et al.*, 2022), was neither detected in our surveys. Finally, regarding the geminivirus ToLCNDV, which since 2012 has rapidly spread in different Spanish and Mediterranean regions (Juárez *et al.*, 2019; Maachi *et al.*, 2022), a low incidence (2.4%) was detected in conventional farming fields, whereas its relative importance increased in a melon field grown under organic farming conditions (33.9%). Since the studied organic field was in the same region as some of the monitored conventional fields, these significant differences might be due to the lack of efficient treatments against *B. tabaci* under organic farming conditions. A general low prevalence of whitefly-transmitted viruses had also been observed in conventional fields monitored in France (Desbiez *et al.*, 2020). However, these results might be affected by the fact that no greenhouses were monitored during the surveys, as it is known that whitefly populations cause major damage under those conditions. Nevertheless, a high prevalence of ToLCNDV (79.69% of infected plants) has already been observed in melon fields located in Murcia, Castilla la Mancha and Alicante (Juárez *et al.*, 2019), which might indicate that this virus could become established in Valencia and other important producing areas during the next years.

Additionally, the molecular diversity of the detected viruses was analyzed to study possible population changes. The phylogenetic analysis of CABYV, MWMV and

ToLCNDV isolates showed that they clustered within their corresponding Mediterranean group, which is consistent with former studies (De Moya-Ruiz *et al.*, 2021; Rabadán *et al.*, 2021). On the other hand, WMV and CMV reflected a different picture, pointing out a dynamic status. Regarding the tripartite cucumovirus, CMV, sequence comparisons revealed that isolates of group II were not present in the studied fields and that isolates of group IA were predominant. This was not surprising, since isolates of group II, which has a lower thermal optimum compared with subgroup I, had only been found in Spain in pepper and tomato fields in Northern areas (Fraile *et al.*, 1997); whereas isolates of group IA had previously been described as prevalent in the Spanish fields (Fraile *et al.*, 1997). Moreover, reassorting isolates IB-IB-IA were found infecting watermelon and melon in Comunidad Valenciana and Andalucía. Traditionally, cucurbit crops had always been infected by isolates classified within IA and II groups, or even with reassorting isolates (IA-II-II), whereas IB isolates, which are more prevalent in Asia (Jacquemond, 2012), were found infecting plants of other families. Nevertheless, during the last years different isolates whose CP genes were classified within the group IB (Xanthis *et al.*, 2015; Ahsan *et al.*, 2020), or even isolates with the three RNA segments that make up the virus genome clustered within that group (IB-IB-IB) (Nagendran *et al.*, 2018), have been found infecting cucurbits in Bulgaria, Greece, Mexico, Pakistan and India. However, these kinds of isolates have not been detected infecting cucurbits in other surveys recently carried out in Europe (Desbiez *et al.*, 2020; Valachas *et al.*, 2021). The types of reassorting isolates (IB-IB-IA) described in this work, had previously been detected infecting a zucchini plant in Eastern Spain in 1995 (Bonnet *et al.*, 2005). However, they were not detected again in subsequent studies. Other studies revealed that reassorting isolates were usually found in a low proportion and that they did not became established in the population, due to a selection against reassortment between IA and IB isolates (Fraile *et al.*, 1997). Taking this into account, further surveys should be made to

determine if isolates of the IB-IB-IA reassortment will be able to become established in this region, if it spreads to new areas and whether it is more virulent or not compared to previously detected isolates.

For WMV, none of the sequenced isolates clustered within the classic group. The `classic´ isolates were already being displaced in Spain in 2005-2006 (Juárez *et al.*, 2013) and, nowadays, they have completely been replaced by the EM groups in other Mediterranean countries (Bertin *et al.*, 2020; Desbiez *et al.*, 2020). The sequencing of both NIb-CP and P3-CI gene regions revealed a high molecular diversity among the WMV isolates, establishing 7 new profiles. The sequencing of the complete genome for every detected profile showed that recombination was frequent among the isolates. Moreover, despite the fact that nonsynonymous mutations were not favored by selection, which is consistent with previous studies (Rabadán *et al.*, 2023), some new mutations were described for the first time in Spanish isolates, specially within the P1 protein coding sequence. This accumulation of changes within the P1 sequence had already been observed in the isolate MeWM7 when compared to isolate Vera (De Moya-Ruiz *et al.*, 2021) and the highest dN/dS ratio was observed within this gene. P1, the first protein of the polyprotein, is the most divergent protein among potyviruses with regard to both length and amino acid sequence. Most of the observed amino acid changes between the sequenced isolates were detected in the N-terminal region of the P1 protein, whereas the C-terminal domain seems to be more conserved. Within this protein, the C-terminal sequence has a well conserved serine protease domain and the N-terminal region is hypervariable, acting as a repressor of the protease activity. This seems to be an evolutionary step to keep viral amplification below host-detrimental levels, to maintain a higher long-term replicative capacity (Pasin *et al*., 2014). Nevertheless, since it has been proposed that P1 protein could strengthen the ability of HC-Pro to suppress RNA silencing and enhance the

pathogenicity of heterologous plant viruses (Valli *et al.*, 2018), it will be necessary to further observe if the detected sequence differences could lead to an increase in symptom severity.

This work shows that, even though the new emerging viruses have not yet spread through the Spanish Mediterranean basin, virus populations can rapidly evolve as a result of mutations and recombination/reassortment events. These evolutionary mechanisms, in addition to long-distance introductions due to plant material exchanges and changing climate conditions, can lead to severe virus epidemics. In this sense, organic farming fields are especially vulnerable since there is a lack of effective treatments against viral vectors. Moreover, these new virus genotypes could overcome the previously described genetic resistances, as previously reported for different viruses (Desbiez *et al*., 2021; Giner *et al*., 2017;). Integrated pest control, good agronomic practices, as well as the development of resistant cultivars will be necessary to improve the phytosanitary status of cucurbits fields, ensuring stable and high-quality production.

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Conflict of interest

The authors declare that there is no conflict of interest.

Supplementary material

Supplementary material can be found at: https://drive.google.com/drive/folders/1OQsVbVaDPjsoBWgpSkd4EqFmtwodcfuU?usp=sharing

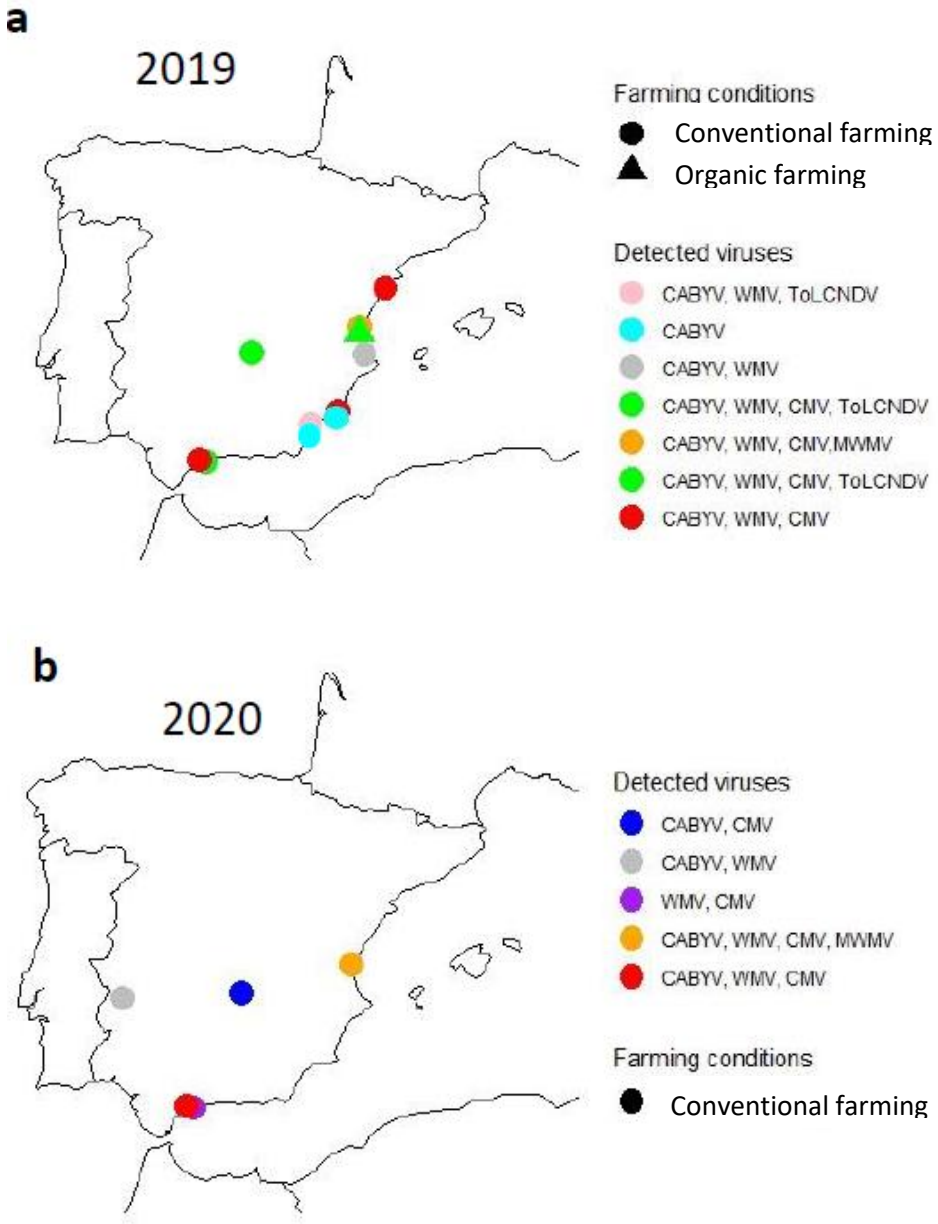

Figure S1. Viruses detected in the different monitored localities during 2019 (a) and 2020 (b). The farming conditions of the monitored fields are indicated.

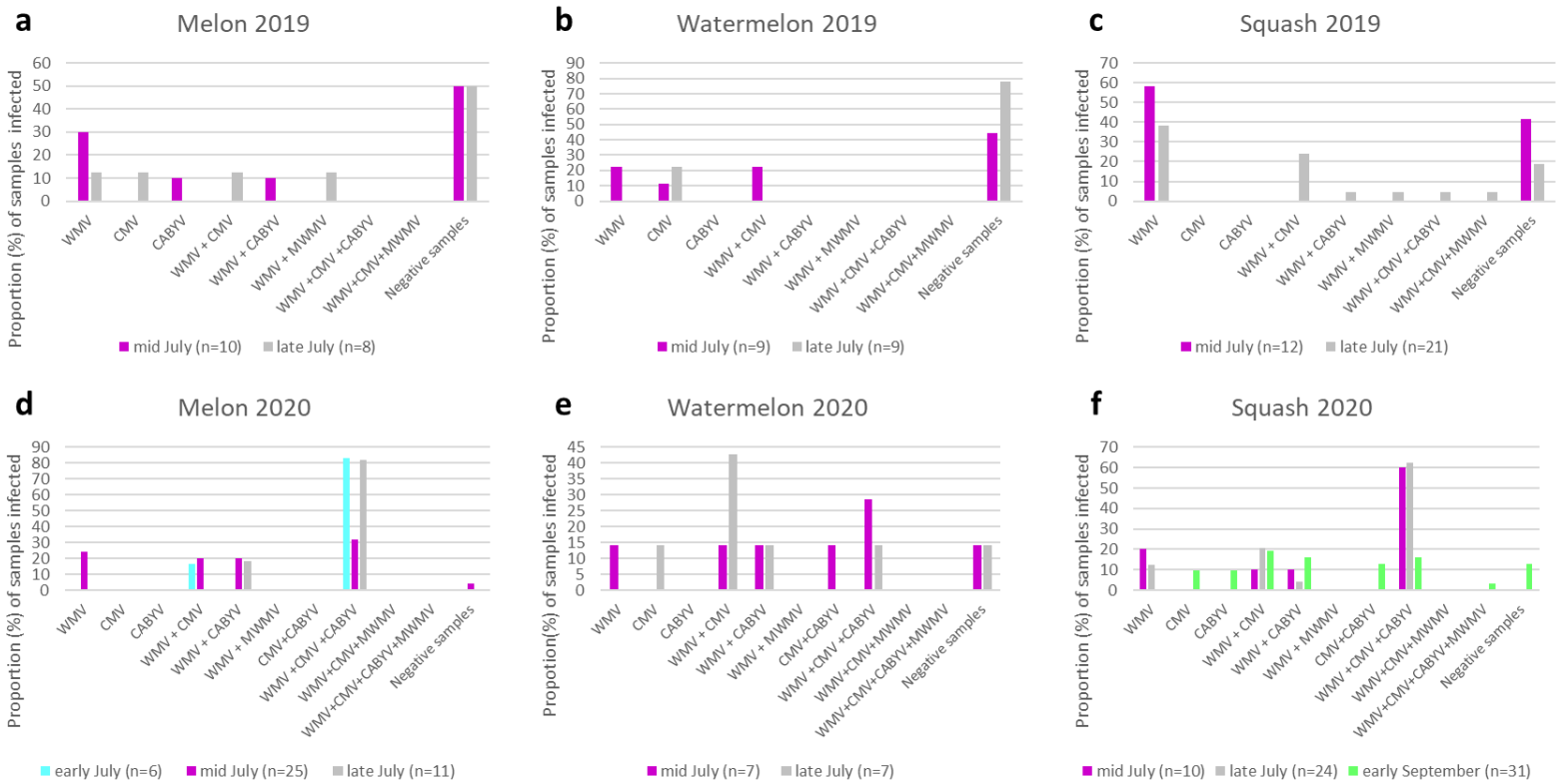

Figure S2. Temporal evolution of single and mixed infections during the summer season in melon (a,d), watermelon (b,e) and squash (c,f) fields located in Museros (Comunidad Valenciana, Spain) during 2019 (a-c) and 2020 (d-f) summer seasons. The survey dates and the number of samples monitored are indicated

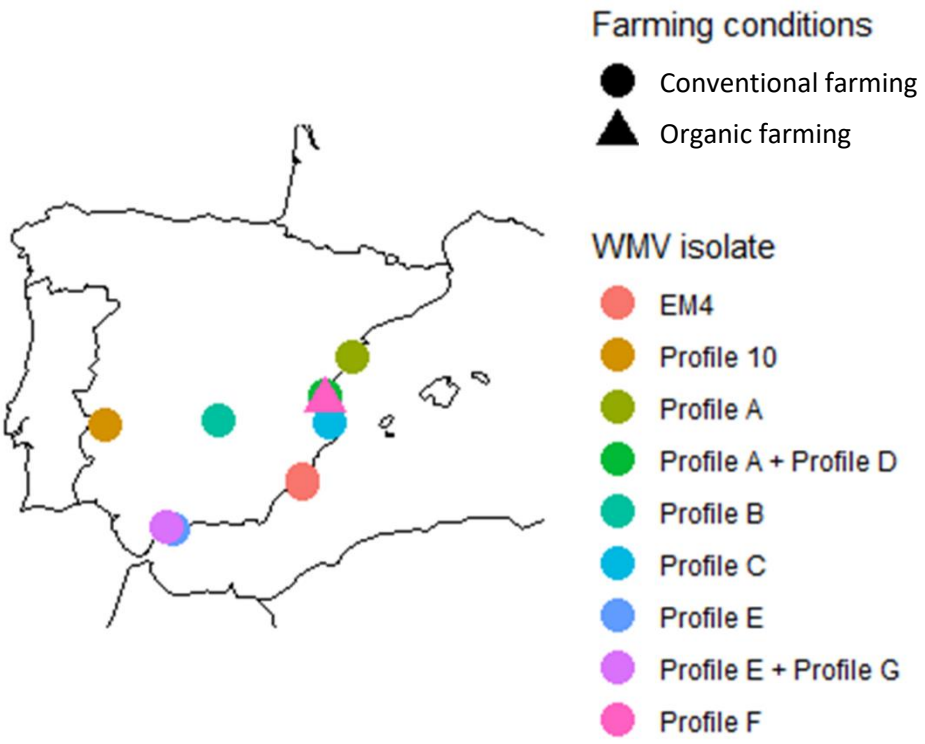

Figure S3. Geographical origin of the different watermelon mosaic virus isolates detected in the surveys. The farming conditions of the monitored fields are also indicated.

a

Location	Profile	Isolate	C. Valenciana	Castilla la Mancha	C. Valenciana	C. Valenciana	Andalucía	C. Valenciana	Andalucía	Extremadura	Región de Murcia	C. Valenciana (2016)	Spain (2019)	France (2000)	France (2003)	France (2004)	France (2005)
	Profile A	EM190221	Profile A	Profile B	Profile C	Profile D	Profile E	Profile F	Profile G	Profile 10	EM4			EM1	EM2	EM3	EM4
			EM190221	EM190182	EM190185	EM190275	EM190179	EM190P93	EM200305	EM200313	EM190155	Vera	MeWM7	FMF00-LL1	FMF03-141	FBR04-37	C05-270
C. Valenciana	Profile A	EM190221															
Castilla la Mancha	Profile B	EM190182	95.8														
C. Valenciana	Profile C	EM190185	94.4	95.0													
C. Valenciana	Profile D	EM190275	94.3	95.5	96.1												
Andalucía	Profile E	EM190179	95.7	90.3	96.5	90.2											
C. Valenciana	Profile F	EM190P93	94.3	95.6	96.5	97.4	90.2										
Andalucía	Profile G	EM200305	93.8	94.8	95.8	96.7	90.4	97.3									
Extremadura	Profile 10	EM200313	92.0	91.4	91.6	91.0	94.3	91.2	90.8								
Región de Murcia	EM4	EM190155	92.5	92.6	94.1	93.5	90.6	93.7	93.7	92.3							
C. Valenciana (2016)		Vera	94.4	95.7	96.6	97.6	95.8	99.3	97.4	91.3	93.8						
Región de Murcia (2019)		MeWM7	94.7	95.6	96.6	98.8	95.9	97.6	97.1	91.3	93.8	97.8					
France (2000)	EM1	FMF00-LL1	94.4	95.8	96.6	97.6	95.7	99.3	97.4	91.4	93.9	99.5	97.9				
France (2003)	EM2	FMF03-141	93.5	93.6	94.6	94.6	95.7	94.7	94.4	91.9	93.9	95.0	94.9	95.0			
France (2004)	EM3	FBR04-37	92.3	91.8	91.8	91.3	96.1	91.6	91.1	96.9	92.6	91.7	91.8	91.8	91.7		
France (2005)	EM4	C05-270	92.6	92.6	94.2	93.6	90.6	93.9	93.8	92.3	99.3	94.0	93.9	94.0	94.1	92.6	
France	Classic Group	WMV-Fr	90.0	89.6	90.4	90.0	91.1	90.5	90.6	90.9	91.1	90.6	90.4	90.6	90.7	91.4	91.2

Figure S4.

b

Location	Profile	Isolate	C. Valenciana	Castilla la Mancha	C. Valenciana	C. Valenciana	Andalucía	C. Valenciana	Andalucía	Extremadura	Región de Murcia	C. Valenciana (2016)	Spain (2019)	France (2000)	France (2003)	France (2004)	France (2005)
	Profile		Profile A	Profile B	Profile C	Profile D	Profile E	Profile F	Profile G	Profile 10	EM4			EM1	EM2	EM3	EM4
		Isolate	EM190221	EM190182	EM190185	EM190275	EM190179	EM190P93	EM200305	EM200313	EM190155	Vera	MeWM7	FMF00-LL1	FMF03-141	FBR04-37	C05-270
C. Valenciana	Profile A	EM190221															
Castilla la Mancha	Profile B	EM190182	97.6														
C. Valenciana	Profile C	EM190185	97.3	98.1													
C. Valenciana	Profile D	EM190275	97.1	98.5	98.8												
Andalucía	Profile E	EM190179	95.2	95.8	96.1	95.6											
C. Valenciana	Profile F	EM190P93	97.4	98.4	99.1	99.1	96.0										
Andalucía	Profile G	EM200305	97.2	98.1	98.5	98.6	95.8	99.0									
Extremadura	Profile 10	EM200313	95.8	95.5	95.6	95.3	97.5	95.5	95.3								
Región de Murcia	EM4	EM190155	96.1	96.0	96.8	96.5	96.2	96.8	96.5	96.7							
C. Valenciana (2016)		Vera	97.5	98.4	99.2	99.1	96.0	99.7	99.0	95.5	96.7						
Región de Murcia (2019)		MeWM7	97.1	98.4	98.7	99.3	95.7	99.1	98.5	95.3	96.5	99.1					
France (2000)	EM1	FMF00-LL1	97.3	98.3	99.0	99.0	95.9	99.6	98.9	95.4	96.7	99.6	99,0				
France (2003)	EM2	FMF03-141	96.9	96.7	97.3	97.1	95.3	97.1	97.0	95.8	96.8	97.2	97.1	97.1			
France (2004)	EM3	FBR04-37	95.9	95.8	96.0	95.6	97.9	95.8	95.7	98.7	97.0	95.8	95.6	95.7	96.1		
France (2005)	EM4	C05-270	96.2	96.1	96.9	96.6	96.2	96.8	96.7	96.7	99.7	96.8	96.5	96.7	96.9	97.0	
France	Classic Group	WMV-Fr	94.5	94.1	95.0	94.6	95.1	94.9	95.0	95.4	95.5	94.8	94.6	94.8	95.0	95.9	95.6

Figure S4. Continuation. Pairwise identities found for the different watermelon mosaic virus profiles sequenced, reference isolates are also included in the analysis. (a) Proportion (%) of identities between nucleotide sequences. (b) Proportion (%) of identity between amino acid sequences. The geographical origin of the samples and the profile in which they were classified is indicated.

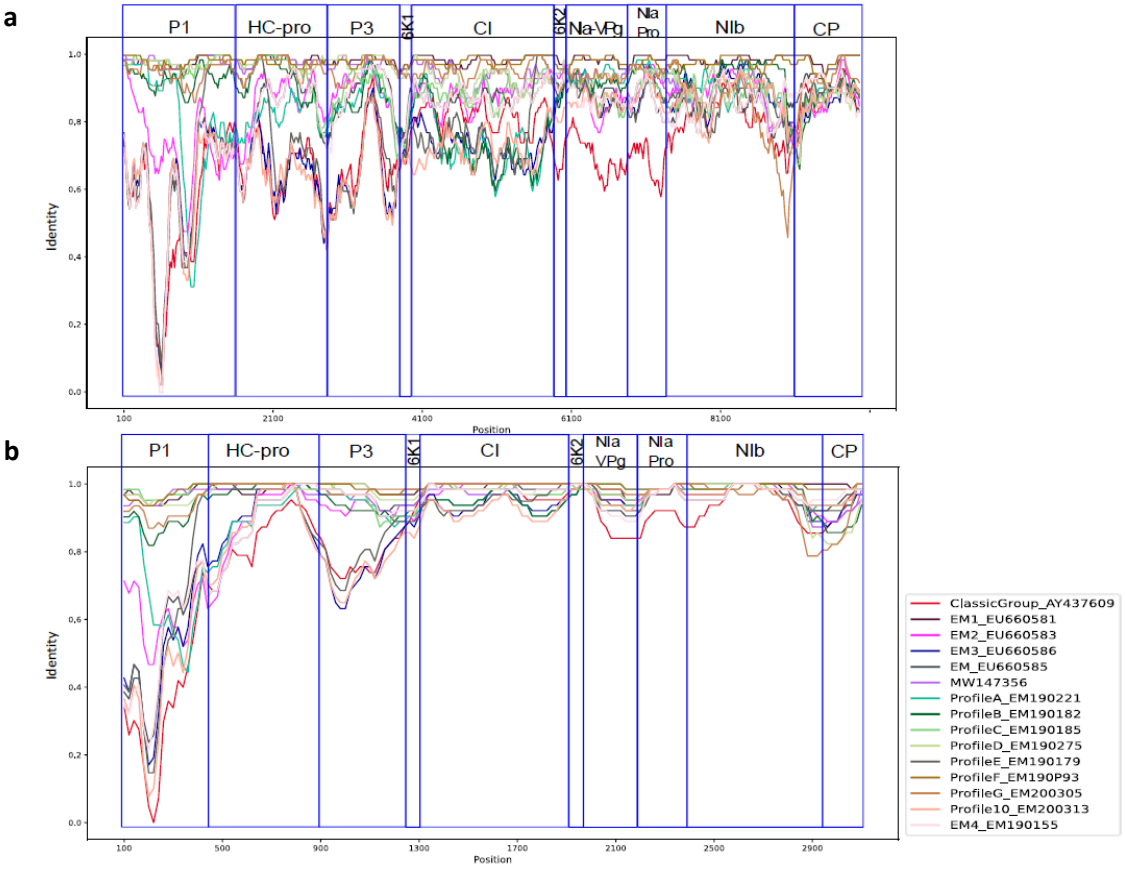

Figure S5. Identity between the sequenced isolates *of* watermelon mosaic virus. Both nucleotide (a) and amino acid (b) sequences were analyzed. The Spanish isolate Vera (MH469650) was used as query. The different potyvirus proteins are indicated. Reference isolates of the previously described molecular groups were included in the analysis.

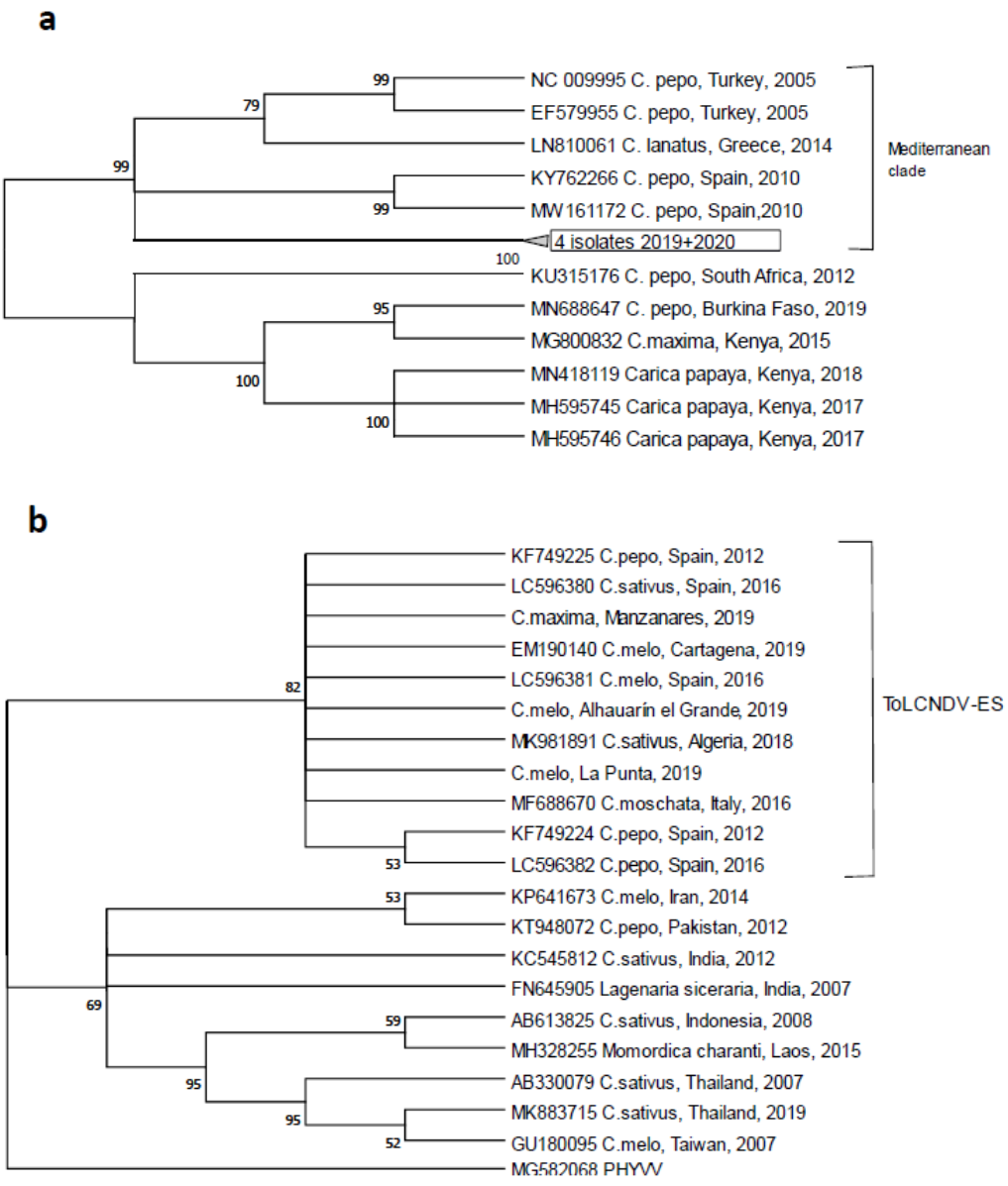

Figure S6. Maximum-likelihood trees obtained for partial nucleotide sequences of the coat protein genes of Moroccan watermelon mosaic virus (MWMV) (a) and tomato lead curl New Delhi virus (ToLCNDV) (b) Bootstrap values (n=500 replicates) above 50% are indicated for each node.

Table S1. Number of plots monitored and samples collected in each locality during 2019 and 2020. Apical leaves of symptomatic plants were collected in open fields cultivated under conventional farming conditions.

			2019		2020	
Region	**Province**	**Locality**	**No. Plots**	**No. Samples**	**No. Plots**	**No. Samples**
C. Valenciana	Valencia	Museros	5	68	6	121
	Valencia	Gandía	1	5		
	Castellón	Benicarló	11	48		
Región de Murcia	Murcia	Torre Pacheco	2	12		
		Cartagena	2	5		
		Pozo de la Higuera	1	3		
Andalucía	Almería	Cuevas del Almanzora	2	2		
	Málaga	Alhaurín el Grande	1	10	1	6
		Coín	4	14	1	2
Castilla la Mancha	Ciudad Real	Manzanares	1	3	1	3
Extremadura	Badajoz	Puebla de la Calzada			3	3
		Total		170		132

Table S2. List of primers sequences used to detect the studied viruses.

Detected virus	Amplified region	Primer name	Primer sequence	Amplicon size (bp)	Reference
WMV	P3-CI	WMV3715F	AWTGAGGATGAGCAAGCWGT	452	Desbiez et al., (2020)
WMV	P3-CI	WMV4166R	TGCAACTGCCTRTGCCACCA		
WMV	NIb-CP	WMV8689F	ACTGAGGCAATTTGTGCAGC	450 or 456	Desbiez et al., (2020)
WMV	NIb-CP	WMV9144R	TATCTTYTGYAGTCGTGGGAC		
CABYV	RdRp	CABYV2316F	CTCCTTCCGATATTGGCTCG	419	Desbiez et al., (2020)
CABYV	RdRp	CABYV2730R	CTCCAGTCAAARCCRGAGCAGTC		
CABYV	CP	CABYV3617F	CGGAAGACGACAACGAAGAA	423	Desbiez et al., (2020)
CABYV	CP	CABYV4040R	CCGTTCCCCTTGTAGAGGAT		
CMV	RNA-1	1CMV703F	CGAYGGYGCKATGATGTTTGAC	376	Desbiez et al., (2020)
CMV	RNA-1	1CMV1076R	AGAGGGGAACCARATRCAATG		
CMV	RNA-2	2CMV1063F	ACCGGGAGYGGTCACMAGAG	446	Desbiez et al., (2020)
CMV	RNA-2	2CMV1510R	TCYCGAAGGCATCTCTGGAA		
CMV	RNA-3	3CMV1468F	CCTTTGCCGAAATTYGATTC	434 or 451	Desbiez et al., (2020)
CMV	RNA-3	3CMV1889R	TGGAYGGACAACCCGTTC		
MWMV	CP	MWMV-CP-F	GATCTTGCCTAGAGTCAGAG	638	
MWMV	CP	MWMV-CP-R	CACTTACGCATGCCCAGGAG		
CYSDV	CP	CYSDV-CP-F	ATGGCGAGTTCGAGTGAGAATAAAACTTCC	756	
CYSDV	CP	CYSDV-CP-R	TCAATTACCACAGCCACCTGGTGC		
CCYV	CP	CCYV-CP-F	ATGGAGAAGACTGACAATAAACAAAACG	752	
CCYV	CP	CCYV-CP-R	TTATTTACTACAACCTCCCGGTGCC		
ZYMV	CP	ZYMV-CP-F	TCAGGCACTCAGCCAACTGTGGC	839	
ZYMV	CP	ZYMV-CP-R	TTACTGCATTGTATTCACACCTAG		
ToLCNDV	DNA-A	ND-A1d	GGGTTGTGAAGGCCCTTGTAAGGTGC	505	
ToLCNDV	DNA-A	ND-A1R	AGTACAGGCCATATACAACATTAATGC		
CGMMV	CP	CGMMV-F	GAAGAGTCCAGTTCTGTTTC	523	
CGMMV	CP	CGMMV-R	ACCCTCGAAACTAAGCTTTC		

Table S3. Molecular profiles of the sequenced isolates of cucumber mosaic virus (CMV)

Crop	Region	Province	Locality	Survey date	Phylogenetic group	Reference isolate in the survey	Accessions[a]
Melon	Andalucía	Málaga	Alhaurín el Grande	12/07/2019	IA-IA-IA		
Melon	Andalucía	Málaga	Alhaurín el Grande	17/06/2020	IA-IA-IA		
Melon	Andalucía	Málaga	Coín	12/07/2019	IA-IA-IA		
Zucchini	Andalucía	Málaga	Coín	17/06/2020	IA-IA-IA		
Watermelon	Murcia	Murcia	Torre Pacheco	11/07/2019	IA-IA-IA		
Melon	Castilla la Mancha	Ciudad Real	Manzanares	12/07/2019	IA-IA-IA		
Melon	Castilla la Mancha	Ciudad Real	Manzanares	23/09/2020	IA-IA-IA		
Squash	C. Valenciana	Castellón	Benicarló	25/07/2019	IA-IA-IA	EM190158	OQ680541, OQ680543, OQ680545
Watermelon	C. Valenciana	Castellón	Benicarló	25/07/2019	IA-IA-IA		
Zucchini	C. Valenciana	Castellón	Benicarló	25/07/2019	IA-IA-IA		
Squash	C. Valenciana	Castellón	Benicarló	25/07/2019	IA-IA-IA		
Watermelon	C. Valenciana	Castellón	Benicarló	25/07/2019	IA-IA-IA		
Melon	C. Valenciana	Valencia	La Punta	26/06/2019	IA-IA-IA		
Squash	C. Valenciana	Valencia	Museros	29/07/2019	IA-IA-IA		
Melon	C. Valenciana	Valencia	Museros	29/07/2019	IA-IA-IA		
Melon	C. Valenciana	Valencia	Museros	01/07/2020	IA-IA-IA		
Squash	C. Valenciana	Valencia	Museros	16/07/2020	IA-IA-IA		
Watermelon	C. Valenciana	Valencia	Museros	29/07/2020	IA-IA-IA		
Squash	C. Valenciana	Valencia	Museros	09/09/2020	IA-IA-IA		
Melon	C. Valenciana	Valencia	Museros	01/07/2020	IB-IB-IA	EM200398	OQ680542, OQ680544, OQ680546
Watermelon	C. Valenciana	Valencia	Museros	16/07/2020	IB-IB-IA		
Melon	C. Valenciana	Valencia	Museros	29/07/2020	IB-IB-IA		
Melon	Andalucía	Málaga	Alhaurín el Grande	24/07/2020	IB-IB-IA		

[a]: GenBank accession number of the different fragments for the reference isolate from the survey. The first accession number refers to the RNA-1 sequenced fragment, the second to the RNA-2 and the third to the RNA-3.

Table S4. Molecular profile of the sequenced isoaltes of watermelon mosaic virus (WMV)

Crop	Region	Province	Locality	Survey date	Phylogenetic group	Reference isolate in the survey	Accessions [a]
Squash	Museros	Valencia	C. Valenciana	29/07/2020	PROFILE A	EM190221	OQ680557, OQ680523, OQ680532
Melon	Museros	Valencia	C. Valenciana	29/07/2020	PROFILE A		
Squash	Museros	Valencia	C. Valenciana	09/09/2020	PROFILE A		
Squash	Museros	Valencia	C. Valenciana	16/07/2020	PROFILE A		
Squash	Museros	Valencia	C. Valenciana	16/07/2020	PROFILE A		
Watermelon	Museros	Valencia	C. Valenciana	16/07/2020	PROFILE A		
Melon	Museros	Valencia	C. Valenciana	01/07/2020	PROFILE A		
Watermelon	Museros	Valencia	C. Valenciana	16/07/2020	PROFILE A		
Squash	Benicarló	Castellón	C. Valenciana	25/07/2019	PROFILE A		
Watermelon	Benicarló	Castellón	C. Valenciana	25/07/2019	PROFILE A		
Watermelon	Benicarló	Castellón	C. Valenciana	25/07/2019	PROFILE A		
Melon	Manzanares	Ciudad Real	Castilla la Mancha	12/07/2019	PROFILE B	EM190182	OQ680558, OQ680524, OQ680533
Squash	Gandía	Valencia	C. Valenciana	23/07/2019	PROFILE C	EM190185	OQ680559, OQ680525, OQ680534
Melon	Museros	Valencia	C. Valenciana	11/07/2019	PROFILE D	EM190275	OQ680562, OQ680528, OQ680537
Squash	Museros	Valencia	C. Valenciana	29/07/2019	PROFILE D		
Squash	Museros	Valencia	C. Valenciana	29/07/2019	PROFILE D		
Squash	Museros	Valencia	C. Valenciana	11/07/2019	PROFILE D		
Melon	Museros	Valencia	C. Valenciana	16/07/2020	PROFILE D		
Squash	Museros	Valencia	C. Valenciana	29/07/2020	PROFILE D		
Watermelon	Museros	Valencia	C. Valenciana	29/07/2020	PROFILE D		
Squash	Museros	Valencia	C. Valenciana	09/09/2020	PROFILE D		

Table S4. Continuation

Crop	Region	Province	Locality	Survey date	Phylogenetic group	Reference isolate in the survey	Accessions[a]
Melon	Alhaurín el Grande	Málaga	Andalucía	12/07/2019	PROFILE E	EM190179	OQ680563, OQ680529, OQ680538
Melon	Alhaurín el Grande	Málaga	Andalucía	24/07/2020	PROFILE E		
Melon	Alhaurín el Grande	Málaga	Andalucía	17/06/2020	PROFILE E		
Zucchini	Coín	Málaga	Andalucía	17/06/2020	PROFILE E		
Watermelon	Coín	Málaga	Andalucía	12/07/2019	PROFILE E		
Zucchini	Coín	Málaga	Andalucía	12/07/2019	PROFILE E		
Squash	Coín	Málaga	Andalucía	12/07/2019	PROFILE E		
Melon	Coín	Málaga	Andalucía	12/07/2019	PROFILE E		
Watermelon	Coín	Málaga	Andalucía	12/07/2019	PROFILE E		
Melon	La Punta	Valencia	C. Valenciana	28/06/2019	PROFILE F	EM190P93	OQ680561, OQ680527, OQ680536
Melon	La Punta	Valencia	C. Valenciana	28/06/2019	PROFILE F		
Melon	La Punta	Valencia	C. Valenciana	26/06/2019	PROFILE F		
Zucchini	Coín	Málaga	Andalucía	17/06/2020	PROFILE G	EM200305	OQ680565, OQ680531, OQ680540
Melon	Cartagena	Murcia	Región de Murcia	11/07/2019	EM4	EM190155	OQ680560, OQ680526, OQ680535
Watermelon	Torre Pacheco	Murcia	Región de Murcia	11/07/2019	EM4		
Melon	Puebla de la Calzada	Badajoz	Extremadura	14/07/2020	PROFILE10	EM200313	OQ680564, OQ680530, OQ680539

[a]: GenBank accession number of the different fragments for the reference isolate from the survey. The first accession number refers to the complete genome sequence, the second to the NIb-CP region and the third to the CI-P3 sequenced fragment.

Table S5. Molecular profile of the sequenced isolates of cucurbit aphid-borne yellows virus (CABYV)

Crop	Region	Province	Locality	Survey date	Reference isolate in the survey	Accessions[a]
Melon	Región de Murcia	Murcia	Torre Pacheco	11/07/2019		
Melon	Región de Murcia	Murcia	Torre Pacheco	11/07/2019		
Melon	Región de Murcia	Murcia	Cartagena	11/07/2019		
Watermelon	Región de Murcia	Murcia	Pozo de la Higuera	11/07/2019		
Melon	Andalucía	Almería	Cuevas del Almanzora	11/07/2019		
Watermelon	Andalucía	Almería	Cuevas del Almanzora	11/07/2019		
Melon	Andalucía	Málaga	Alhaurín el Grande	12/07/2019		
Melon	Andalucía	Málaga	Alhaurín el Grande	17/06/2020		
Melon	Andalucía	Málaga	Coín	12/07/2019		
Watermelon	Andalucía	Málaga	Coín	12/07/2019		
Zucchini	Andalucía	Málaga	Coín	12/07/2019	EM190135, EM190173, EM190202, EM190187	OQ680549, OQ680553, OQ680550, OQ680554, OQ680551, OQ680555, OQ680552, OQ680556
Melon	Castilla y la Mancha	Ciudad Real	Manzanares	12/07/2019		
Melon	Extremadura	Badajoz	Puebla de la Calzada	14/07/2020		
Watermelon	Extremadura	Badajoz	Puebla de la Calzada	14/07/2020		
Watermelon	C. Valenciana	Castellón	Benicarló	25/07/2019		
Zucchini	C. Valenciana	Castellón	Benicarló	25/07/2019		
Squash	C. Valenciana	Valencia	Gandía	23/07/2019		
Melon	C. Valenciana	Valencia	La Punta	01/07/2019		
Watermelon	C. Valenciana	Valencia	Museros	29/07/2019		
Squash	C. Valenciana	Valencia	Museros	29/07/2019		
Melon	C. Valenciana	Valencia	Museros	01/07/2020		
Melon	C. Valenciana	Valencia	Museros	16/07/2020		
Watermelon	C. Valenciana	Valencia	Museros	16/07/2020		
Melon	C. Valenciana	Valencia	Museros	29/07/2020		
Squash	C. Valenciana	Valencia	Museros	29/07/2020		
Watermelon	C. Valenciana	Valencia	Museros	29/07/2020		
Squash	C. Valenciana	Valencia	Museros	09/09/2020		

[a]: GenBank accession number of the different fragments for the reference isolate from the survey

Table S6. Molecular profile of the sequenced isolates of Moroccan watermelon mosaic virus (MWMV)

Crop	Region	Province	Locality	Survey date	Reference isolate in the survey	Accessions[a]
Squash	C. Valenciana	Valencia	Museros	29/07/2019	EM190284	OQ680548
Melon	C. Valenciana	Valencia	Museros	29/07/2019		
Melon	C. Valenciana	Valencia	La Punta	28/06/2019		
Squash	C. Valenciana	Valencia	Museros	09/09/2020		

[a]: GenBank accession number of the different fragments for the reference isolate from the survey

Table S7. Molecular profile of the sequenced isolates of tomato leaf curl New Delhi virus (ToLCNDV)

Crop	Region	Province	Locality	Survey date	Reference isolate in the survey	Accessions[a]
Melon	Región de Murcia	Murcia	Cartagena	11/07/2019	EM190140	OQ680547
Melon	Andalucía	Málaga	Alhaurín el Grande	12/07/2019		
Squash	Castilla y la Mancha	Ciudad Real	Manzanares	12/07/2019		
Melon	C. Valenciana	Valencia	La Punta	05/08/2019		
Melon	C. Valenciana	Valencia	La Punta	06/08/2019		

[a]: GenBank accession number of the different fragments for the reference isolate from the survey

Table S8. List of primers used to sequence the complete genome of watermelon mosaic virus.

Forward Primer	Sequence	Reverse primer	Sequence	Expected product (bp)
WMV-1-F	ACAACTCATAAAGACATCAAAC	WMV-1-R	GCTTCATTGCCTCATCCATC	723
WMV-2-F	AGGGTGATTTCTGTACCCAGA	WMV-2-R	GAGTGAATTTTGGTATAACAGC	771
WMV-3-F	TATGCTCGTATGAACAAGC	WMV-3-R	GCTGCCAATTCACCACATTG	755
WMV-4-F	AGTGGGTTACTGTTTGATGA	WMV-4-R	TTCTTGTCCAACTGATTATC	732
WMV-5-F	CATATGCTGCAGATACAGGA	WMV-5-R	ACATCAAACATTGTCGGCCA	765
WMV-6-F	GCAAAGGAAGGATATTGCTA	WMV-6-R	GAGTAAACCTTTTCAATTGCC	905
WMV-7-F	TGTCCACAATCAGCACATTC	WMV-7-R	TGCGACAATCTTCTCAAGTTG	760
WMV-8-F	GAGGATGAGCAAGCTGTGGTGC	WMV-7-R	CCACTCGTCATAACAACTAT	771
WMV-9-F	TTTCACGAGAGACAGCAGC	WMV-9-R	GTCACTCCATTTTCAATGAT	755
WMV-10-F	GAGAAGCACTACAAAGT	WMV-10-R	CCAAAACCTGCATCACACTT	750
WMV-11-F	TCAGAAATGATGCTACACAA	WMV-11-R	CATCTCTTCACTGAGTAAATG	310
WMV-12-F	AAGTGTGATGCAGGTT	WMV-12-R	TCAACTCCATACATGTGTAT	720
WMV-13-F	TACACCATGGAACATACTTT	WMV-13-R	CTGAGACTGTTGCGCGTAA	866
WMV-14-F	ACAATGTTTGGAGTTGGGTA	WMV-14-R	TCAGCTTGCCCACAAGC	702
WMV-15-F	GGTATACATGGATTAACATC	WMV-15-R	GCCAACTGTCCATGGACATTT	904
WMV-16-F	TGTTGTACCTTAGCTGTG	WMV-16-R	CCATCAACTAGCATGGCTTGGTG	758
WMV-17-F	GCTGTAAACAAGGTTGGTC	WMV-17-R	CTTCAAGGTATCTTTGC	521
WMV-18-F	CTGAGGCAATTTGTGCAGC	WMV-18-Rbis	CCTTTCCTGCATCCAAAT	304
WMV-19-F	CACAGTTTGAATCATGGTA	WMV-19-R	GACAACAAACATTACCGTAC	793

Table S9. Worldwide isolates used to construct the CMV phylogenetic trees. The names of the isolates and their GenBank accession number for each RNA fragment are indicated.

	GenBank accession number		
Isolate	**RNA-1**	**RNA-2**	**RNA-3**
M160191	MN990924	MN990928	MN990932
EM170683	MN990925	MN990929	MN990933
EM170589	MN990926	MN990930	MN990934
EM160348	MN990927	MN990931	MN990935
Cah1	FJ268744	FJ268745	FJ268746
New Delhi	GU111227	GU111228	GU111229
Tfn	Y16924	Y16925	Y16926
Iizuka	D16403	D16406	D16405
Pl-1	AM183114	AM183115	AM183116
Phy	DQ402477	DQ412731	DQ412732
Fny	D00356	D00355	D10538
NT9	D28778	D28779	D28780
LY	AF198101	AF198102	AF198103
Vir	HE962478	HE962479	HE962480
IRN-SisSh64	LC066474	LC066475	LC066476
TUR81	LC066504	LC066505	LC066506
SFQT1-2	HQ283392	HQ283391	HQ283393
Mf	AJ276479	AJ276480	AJ276481
S	Y10884	Y10885	
LS	AF416899	AF416900	
Trk7	AJ007933	AJ007934	
Q-CMV	X02733	X00985	
CLW2	JN054636		
SKO20ST2	OL472038		
Z1	GU327366		
O		D10209	
Y		D12538	
CTL		EF213024	
ER			U15730
RT88			AJ810257
RT52			AJ810258
LiTW			AJ131619

Table S9. Continuation

	GenBank accession number		
Isolate	**RNA-1**	**RNA-2**	**RNA-3**
Taean			JX415272
D			AJ131624
Sny			U66094

Table S10. Recombination breakpoints in WMV isolates calculated by different algorithms implemented in RDP.4.0 (see section 2.4 for the description).

Begin	End	Recombinant sequence (s)	Minor parental sequence (s)	Major parental sequence (s)	Detection methods and their *p-values*						
					RDP	GENECONV	BootScan	Maxchi	Chimaera	SiSscan	3Seq
4,281	5,637	EM190221/ EM190182	FBR04-37	EM190275	$5.79x10^{-15}$	NS	NS	$1.28X10^{-10}$	$5.08X10^{-11}$	$1.18X10^{-08}$	$1.54X10^{-16}$
1,831	3,775	EM190185/ EM190275	CHI87-620	FBR04-37	$4.86X10^{-05}$	NS	NS	$6.20X10^{-04}$	$1.68X10^{-05}$	$1.03X10^{-12}$	$2.01X10^{-03}$
1,137	2,027	EM190179	EM190182	FBR04-37	$1.47X10^{-34}$	$9,26X10^{-29}$	NS	$3.33X10^{-14}$	$2.25X10^{-14}$	$7.17X10^{-12}$	$1.83X10^{-14}$
1,950	2,344	EM190155	FMF03-141	FBR04-37	$4.18X10^{-10}$	NS	NS	$3.17X10^{-05}$	$7.95X10^{-04}$	NS	$7.94X10^{-08}$

*NS: not significative

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Chapter 2.

Melon Genome Regions Associated with TGR-1551-Derived Resistance to Cucurbit yellow stunting disorder virus.

Chapter 2. Melon genome regions associated with TGR-1551 derived resistance to cucurbit yellow stunting disorder virus.

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Abstract

Cucurbit yellow stunting disorder virus (CYSDV) is one of the main limiting factors of melon cultivation worldwide. To date, no commercial melon cultivars resistant to CYSDV are available. The African accession TGR-1551 is resistant to CYSDV. Two major quantitative trait *loci* (QTL) have been previously reported, both located near each other in chromosome 5. With the objective of further mapping the gene or genes responsible of the resistance, a recombinant inbred lines (RILs) population derived from the cross between TGR-1551 and the susceptible cultivar 'Bola de Oro' was evaluated for resistance to CYSDV in five different assays, and genotyped in a genotyping by sequencing (GBS) analysis. The major effect of one of the two QTLs located on chromosome 5 was confirmed in the multi-environment RILs assay and additionally verified through the analysis of three segregating BC_1S_1 populations derived from three resistant RILs. Furthermore, progeny test using the offspring of selected BC_3 plants allowed the narrowing of the candidate interval to a 700 kb region. The SNP markers identified in this work will be useful in marker-assisted selection in the context of introgression of CYSDV resistance in elite cultivars.

Keywords: CYSDV; QTLs; molecular markers

1. Introduction

The cucurbit yellow stunting disorder virus (CYSDV) is a *Crinivirus* of the family *Closteroviridae* (Martelli *et al.*, 2002). The most important cucurbit crops are infected by this virus, and melon (*Cucumis melo* L.) is one of the species most severely affected.

CYSDV was first reported in the United Arab Emirates (Hassan & Duffus, 1990) and subsequently spread throughout the Middle East and the Mediterranean Basin (Abou-Jawdah *et al.*, 2000; Célix *et al.*, 1996; Desbiez *et al.*, 2000; Kao *et al.*, 2000; Louro *et al.*, 2000; Abou-Jawdah *et al.*, 2000; Yakoubi *et al.*, 2007), North and Central America (Brown *et al.*, 2007; Kao *et al.*, 2000; Kuo *et al.*, 2007; Polston *et al.*, 2008), and China (Liu *et al.*, 2010). Nowadays, this virus has become a significant threat throughout the cucurbit production regions in the southern United States, Mexico, and Central America (Tzanetakis *et al.*, 2013), and has been reported as the most economically important of the viruses affecting cucurbit production in the southwestern United States (Wintermantel *et al.*, 2017).

Although initially thought to be restricted to members of the family *Cucurbitaceae*, more recent studies have demonstrated that CYSDV can infect species from at least

nine plant families (Wintermantel *et al.*, 2009). CYSDV is exclusively transmitted by the sweet potato whitefly, *Bemisia tabaci*, in a semipersistent manner (Célix *et al.*, 1996). Although the virus can be transmitted by individual whiteflies, feeding by large numbers of viruliferous insects improves transmission rates (Célix *et al.*, 1996). To date, artificial infection methods such as mechanical inoculation or agroinfiltration have not been successful.

The virus can often remain latent for a relatively long period, up to three to four weeks after infection (Wisler *et al.*, 1998). The infection appears with spotted/mottled symptoms early followed by extensive interveinal chlorosis. As with other criniviruses, symptoms are more prominent on older leaves with younger leaves remaining symptomless. CYSDV infections result in reduced plant vigor (Tzanetakis *et al.*, 2013). The main damage is produced if the virus infects young plants, which can cause an important yield reduction in terms of fruit number and weight (Brown *et al.*, 2007; López-Sesé & Gómez-Guillamón, 2000). Symptoms in fruits are not obvious, but a decrease in sugar levels in CYSDV-infected plants has been also reported (Abou-Jawdah *et al.*, 2000; López-Sesé & Gómez-Guillamón, 2000). These effects on yield and quality cause important economic losses in many areas.

So far, the main strategy to limit the incidence of vector-borne viral diseases has been the application of insecticides to reduce vector populations, combined with selected cultural practices. However, environmental concerns and the ability of the vectors to develop insecticide resistance, make necessary the development and deployment of strategies that do not rely on chemicals. Genetic resistance combined with cultural practices could become a viable means to increase yields in crops produced in open fields despite the presence of viruses (Lapidot *et al.*, 2014).

Resistance to CYSDV in melon was first reported in the African accession TGR-1551 (López-Sesé & Gómez-Guillamón, 2000). Later, the Indian accession PI 313970 was also described as resistant, although resistance was partial as plants showed late symptoms (McCreight *et al.*, 2008). Resistance from PI 313970 has been reported as a monogenic recessive (Mccreight & Wintermantel, 2011).

TGR-1551 is tolerant to the vector, *Bemisia tabaci* (Soria *et al.*, 1999), and resistant to the virus itself (López-Sesé & Gómez-Guillamón, 2000). Resistance mechanism to the virus in TGR-1551 has been shown to involve a restriction of the virus movement in the vascular system and/or prevention of high levels of virus accumulation (Marco *et al.*, 2003). The initial segregation analyses in the family produced from the cross between TGR-1551 and the susceptible Spanish cv. Piel de

Sapo, suggested a monogenic dominant control of the resistance (López-Sesé & Gómez-Guillamón, 2000). Other studies proposed a codominant or more complex nature of the resistance (Park *et al.*, 2007). Subsequent analysis revealed differences in TGR-1551 response to different CYSDV isolates (Gómez-Guillamón *et al.*, 2002). Moreover, the evaluation date has an effect of the CYSDV response as for example later assessment dates result in high symptom scores in the heterozygotes, so suggesting a recessive control of the resistance (McCreight *et al*., 2015). Recent work suggested that the resistance from TGR-1551 and PI 313970 may be allelic (McCreight *et al.*, 2019).

So far, there are no commercial cultivars resistant to CYSDV. On one hand, the nature of the exotic resistant germplasm, of poor agronomic value, makes it difficult the introgression of resistance into a commercial background. Moreover, phenotyping for resistance in the context of breeding programs is challenging. Besides the bias caused by plants that escape whitefly infection, the difficulty in discriminating among viruses based on symptoms is a characteristic of criniviruses (Abrahamian & Abou-Jawdah, 2014). Yellowing symptoms can also be confused with nutritional disorders or phytotoxicity, among others (Wisler *et al.*, 1998). As a consequence, differentiation requires detection test, preferably sensitive nucleic acid detection methods (Abrahamian & Abou-Jawdah, 2014). Protocols for quantification of CYSDV viral titer are available, based on qRT-PCR (Gil-Salas *et al.,* 2007). The most frequent approach in breeding programs for resistance to CSYDV is the use of controlled inoculations mediated by *B. tabaci*, accompanied by diagnosis by molecular tests.

The availability of molecular markers for their use in marker assisted selection (MAS) allows avoiding the difficulties aforementioned. In the case of CYSDV, two main quantitative trait *loci* (QTLs), both located near in chromosome 5, have been reported as linked to resistance derived from TGR-1551 (Palomares-Ríus *et al.*, 2016, 2018). However, further mapping of the gene or genes responsible for the resistance is needed, in order to dispose of markers suitable for MAS.

The objective of this work was to better depict the genetic architecture of the CYSDV resistance derived from TGR-1551, with the aim of identifying molecular markers useful for MAS in breeding programs directed to introgress this resistance into elite cultivars. To attain this objective, several populations, a RIL population and derived advanced backcrosses, obtained from an original cross between TGR-1551 and the highly CYSDV susceptible Spanish cultivar 'Bola de oro' have been genotyped and evaluated for CYSDV resistance.

2. Results

2.1. Multi-environment phenotyping for resistance to CYSDV of the RIL population

The RIL population (F7/F8 generations) derived from the cross TGR-1551 x 'Bola de Oro' (BO) was evaluated for resistance to CYSDV in five different years. Disease assessment was carried out by the evaluation of symptom development in all the assays. Additionally, the estimation of virus titer in plants by qRT-PCR was done in 2012 and 2013 (**Supplementary Table S1**).

All BO plants showed symptoms in all the assays, whereas TGR-1551 plants remained asymptomatic. Plants of the F_1 showed slight symptoms, later and less severe than those observed in BO plants. Viral accumulation was not detected in the resistant parent, TGR. The virus titer in BO was either significantly higher than in the F_1 or similar, depending on the assay (**Supplementary Table S1**).

Correlations between symptom scores in the different years were moderate to high and highly significant in all cases (values between 0.367 and 0.839, $p<0.005$ in all comparisons, **Supplementary Table S2**). Correlations between virus titer values measured by qRT-PCR in 2012 and 2013 assays were high and highly significant in all comparisons (0.750-0.824, $p<0.0001$, **Supplementary Table S2**). In 2012 and 2013 it was possible to relate symptoms and virus titer. Correlation between symptom score and virus titer was high and highly significant for both years (0.513-0.776, $p<0.0001$, **Supplementary Table S2**). The severity of symptoms does not always correlate with virus titer (Pallas & García, 2011). However, a significant correlation between symptom development and viral accumulation has been reported in melon viral diseases caused by viruses of different families, such as the geminivirus tomato leaf curl New Delhi virus (Sáez *et al.*, 2017) or the potyvirus watermelon mosaic virus (Díaz-Pendón *et al.*, 2005). Concretely, in the case of CYSDV previous studies found a relationship between the time of symptoms appearance and viral accumulation, with delayed development in plants with lower titers (Wintermantel *et al.*, 2016). A positive correlation between CYSDV viral titer and whitefly transmission in cucurbits host has been reported (Wintermantel *et al.*, 2016). Thus, the fact that lower symptoms found in TGR-1551-derived resistant plants correspond to lower viral titers is epidemiologically important in the reduction of inoculum sources in fields.

The effect of genotype (G), environment (E), and the interaction between them (GxE) was explored with data of the 46 coincident RILs included in the 2009, 2010, 2012, and 2013 assays. There was a significant effect of both, genotype and

environment, and also of the interaction between them ($p<0.0001$). Although differences among RILs were observed due to the effect of the interaction GxE, as an average, symptoms were significantly lower in 2009. On the contrary, symptom scores were significantly higher in 2012. *B. tabaci* confined in clip-cages was the inoculation method used in 2009 and, once inoculated, plants were kept in pots in a glasshouse. It seemed that the cultivation and growing systems led to lower symptom development. Massive inoculation was used in 2010, 2012 and 2013, with plants growing in plastic greenhouse. The highest temperatures were registered in 2012 (data not included), which could explain the higher symptoms observed in this assay.

Resistance to CYSDV in TGR-1551 has been reported as associated with restriction of the virus movement and/or reduction in virus replication (Marco *et al.*, 2003), which could be limiting the cascade of events associated with symptom induction in TGR-derived resistant plants (Maule *et al.*, 2000). Our results show that despite the high correlation between symptoms and viral accumulation, there are symptomless plants in which virus is detected and, also, plants with symptoms of yellowing that could be attributed to CYSDV in which the virus cannot be detected in the moment of the analysis. The accumulation of the particles and restriction of the virus movement in TGR-1551 has previously been reported (Marco *et al.*, 2003; McCreight *et al.*, 2019), which could explain the virus titer found in symptomless plants. On the other hand, as previously stated, yellowing symptoms caused by CYSDV are sometimes difficult to visually distinguish, not only from symptoms produced by other criniviruses, but also from nutritional disorders or phytotoxicity (Wisler *et al.*, 1998; Abrahamian & Abou-Jawdah, 2014), which can explain the occurrence of symptomatic plants with a negative qRT-PCR. All these factors condition the evaluation of resistance against CYSDV and highlight the need to use efficient disease assessment methodologies in breeding programs. In this work, the availability of evaluation data (both symptoms and virus titer) for most of the RIL lines (several plants per RIL) allowed and accurate classification of each RIL as resistant or susceptible.

2.2. QTL analysis with the RIL population

A QTLs analysis was performed using the GBS1 and phenotypic data of the RILs evaluated in each assay. The map used was the SNPs map previously constructed with the GBS1 data from the RILs (Pérez-de-Castro *et al.*, 2019). Chromosome 5 systematically appeared in all the assays as involved in resistance to CYSDV (**Table 1**). Two genomic regions were identified in this chromosome, which explained

variation in symptoms severity and virus titer detected by qRT-PCR, respectively. The QTL associated with symptoms severity explained between 27 % and 62 % of the variance for this trait and, considering the intervals defined for each assay, spanned between 6,810,744 and 24,296,585 bp. For virus titer detected by qRT-PCR, the QTLs identified explained between 49 % and 53 % of the variance, spanning between 24,791,006-27,121,114 bp) (**Table 1**). Both intervals were near a region for which polymorphic markers were not available from the GBS1, of approximately 10 cM and 1.1 Mb (between 66.4 and 75.9 cM, from 25,229,866 to 26,193,386 bp) (**Figure 1**). This would possibly interfere with the results of the QTL analyses.

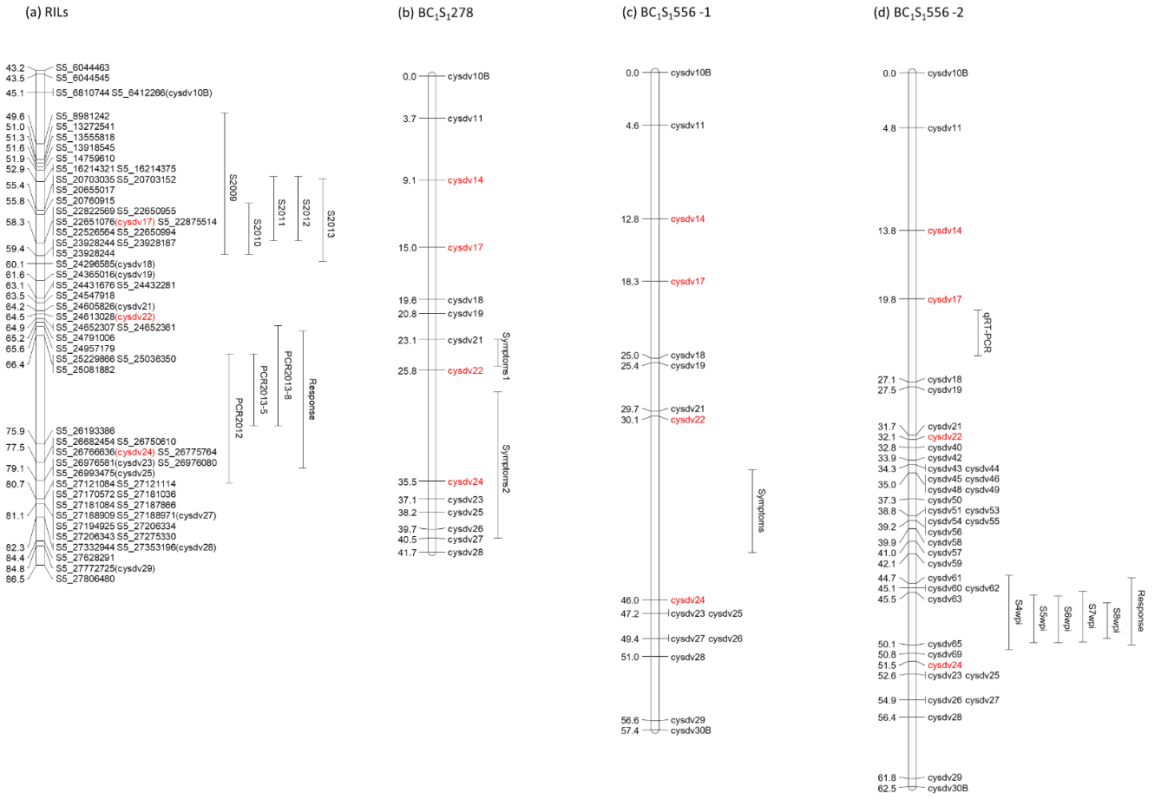
As aforementioned, considering evaluation data gathered in all the assays, each RIL was classified as resistant or susceptible. The QTL obtained with this phenotypic value (response in **Table 1**) explained 44 % of the variance, with the interval extending from 24,957,179 to 26,993,475 bp. This interval included the 10 cM region lacking markers (**Figure 1**), which could account for the size of the interval.

Table 1. Quantitative trait loci (QTLs) identified in chromosome 5 in the different assays with the recombinant inbred lines (RILs) population derived from the cross between TGR-1551 and the cultivar 'Bola de Oro', phenotyped for resistance to cucurbit yellow stunting disorder virus and genotyped by sequencing.

Trait [1]	Interval [2]	Nearest marker [3]	Kruskal-Wallis		Composite interval mapping	
			Mean BO [4]	Mean TGR [5]	LOD [6]	R^2 [7]
S2009 (Scale 0-9)	46.9-59.3 cM 6,810,744-23,928,244 bp	*S5_16214321*	4.93	2.65	6.4	0.27
S2010 (Scale 0-9)	54.8-59.3 cM 16,214,321-23,928,244 bp	*S5_22526564*	6.70	2.09	18.5	0.62
S2011 (Scale 0-9)	52.5-58.1 cM 14,759,610-22,875,514 bp	*S5_16214375*	6.16	0.71	17.9	0.47
S2012 (Scale 0-5)	52.5-58.1 cM 14,759,610-22,875,514 bp	*S5_16214375*	4.26	1.89	12.3	0.34
S2013 (% infection)	52.7-59.9 cM 14,759,610 -24,296,585 bp	*S5_20703035*	43.26	19.27	10.5	0.30
qRT-PCR2012	68.8-79.3 cM 25,036,350 -27,121,114 bp	*S5_26193386*	28.26	35.17	16.1	0.49
qRT-PCR2013-5	68.0-74.3 cM 25,036,350 -26,193,386 bp	*S5_26193386*	26.88	35.06	19.9	0.53
qRT-PCR2013-8	65.5-74.3 cM 24,791,006 -26,193,386 bp	*S5_25229866*	26.09	33.08	15.1	0.51
Response	66-78 cM 24,957,179-26,993,475 bp	*S5_25229866*	0.94	0.33	12.7	0.44

[1] Trait: symptom evaluation for the five assays (S2009, S2010, S2011, S2012, S2013, scale for evaluated is indicated for each assay), qRT-PCR virus titer in 2012 (qRT-PCR2012) and 2013 (two evaluation dates: 5 weeks post inoculation, qRT-PCR2013-5, and 8 weeks post inoculation, qRT-PCR2013-8) and response (classification of each RIL as resistant or susceptible considering evaluation data gathered in all the assays). See Materials and methods section for details. [2] Interval position of the putative QTL on the genetic and the physical map according to a LOD drop of 2. The physical position (v3.6.1) is defined by the position of the markers flanking the QTL interval. [3] Closest marker to the LOD peak. Significance level in the Kruskal-Wallis test was 0.0001 for all the markers. [4] Mean of the genetic class TGR-1551 for the corresponding marker. [5] Mean of the genetic class 'Bola de Oro' for the corresponding marker. [6] Higher logarithm of the odds score. [7] Percentage of phenotypic variance explained by the QTL.

Figure 1. Map of the region of chromosome 5 involved in resistance to CYSDV generated with data from: (a) The RILs population genotyped by GBS; (b) The BC_1S_1278 population genotyped with the marker set CYSDV1; (c) The BC_1S_1556 population genotyped with the marker set CYSDV1; (d) The BC_1S_1556 population genotyped with the marker sets CYSDV1 and CYSDV2. The QTLs obtained in each of the assays are represented by bars. Markers defining the most important QTLs are indicated in red.



2.3. Phenotyping for resistance to CYSDV and QTL analysis in three selected BC_1S_1 progenies derived from selected resistant RILs

Three of the most resistant and vigorous RILs, evaluated in most of the assays, RIL 278, RIL556, and RIL110, were selected to produce segregating generations (**Supplementary Figure S1**).

These RILs were backcrossed to the susceptible parent, BO, and one plant from each of the BC_1 generations was selfed to generate the BC_1S_1 populations. The selection of the resistant RILs was done before the GBS1 was available. GBS results of the RILs confirmed later that RIL110 and RIL556 were both homozygous for the TGR allele for the whole chromosome 5 (**Supplementary Table S3**). RIL278 was also TGR homozygous for almost the whole chromosome 5, with the exception of the region below 27,628,291, for which it was homozygous for BO alleles.

BC_1S_1 plants derived from RIL278 and RIL556 were phenotyped for resistance in 2016. Plants of the susceptible parent BO started showing symptoms from the first evaluation dates, which increased with time (**Figure 2**). Viral accumulation was confirmed in all plants by qRT-PCR. Some plants of the resistant parent, TGR-1551, showed slight spotting, although later was confirmed by qRT-PCR that was not caused by the presence of virus (**Supplementary Table S1**). Similarly, the yellowing observed in some evaluation dates in plants of the RILs 278 and 556 could not be attributed to the virus. Additionally to the parents, some plants of each BC_1 generation (selected RIL x BO) were evaluated. BC_1 plants showed milder symptoms than BO at all evaluation dates for both RILs. The first symptoms in the BC_1 derived from RIL556 appeared delayed with respect to BO. The virus was detected in all BC_1 plants but at lower levels than in BO.

The first set of markers (derived from the RILs GBS) was designed for its use in the Agena Bioscience genotyping platform (CYSDV1, **Supplementary Table S4**). This set included markers covering the genomic regions of the two QTLs obtained with the RILs analysis. The QTL analysis was performed with symptom evaluation in the 133 $BC_1S_1$278 plants and the 134 $BC_1S_1$556 plants. (**Table 2**). A QTL was identified for both families in the region around markers *cysdv22* (24,613,028 bp) and *cysdv24* (26,766,636 bp), which explained in both families 21 and 58% of the variation for symptom evaluation, respectively (**Figure 1, Table 2**). These results confirmed the QTLs positions found in the RILs populations but supported the need to saturate the region between markers *cysdv22* and *cysdv24*.

Table 2. Quantitative trait loci (QTLs) identified in chromosome 5 in BC_1S_1 progenies of RILs 278 and 556 derived from the cross between TGR-1551 and the cultivar 'Bola de Oro', phenotyped for resistance to cucurbit yellow stunting disorder virus and genotyped with markers set CYSDV1 (see text for description).

Trait [1]	**Interval** [2]	**Nearest marker** [3]	**Composite interval mapping**	
			LOD [4]	R^2 [5]
Symptoms 278	23.1-25.4 cM 24,605,826- 24,613,028 bp	*cysdv22*	6.8	0.21
	27.7-40.5 cM 24,613,028-27,188,971 bp	*cysdv24*	4.5	0.12
Symtpoms 556	40.0-48.0 cM 24,613,028-26,993,475 bp	*cysdv22*	25.5	0.58

[1] Trait: Symptom evaluation in the different progenies, $BC_1S_1$278 (Symptoms 278) and $BC_1S_1$556 (Symptoms 556). [2] Interval position of the putative QTL on the genetic and the physical map according to a LOD drop of 2. The physical position (v.3.6.1) is defined by the position of the markers flanking the QTL interval. [3] Closest marker to the LOD peak. Significance level in the Kruskal-Wallis test was 0.0001 for all the markers. [4] Higher logarithm of the odds score.[5] Percentage of phenotypic variance explained by the QTL

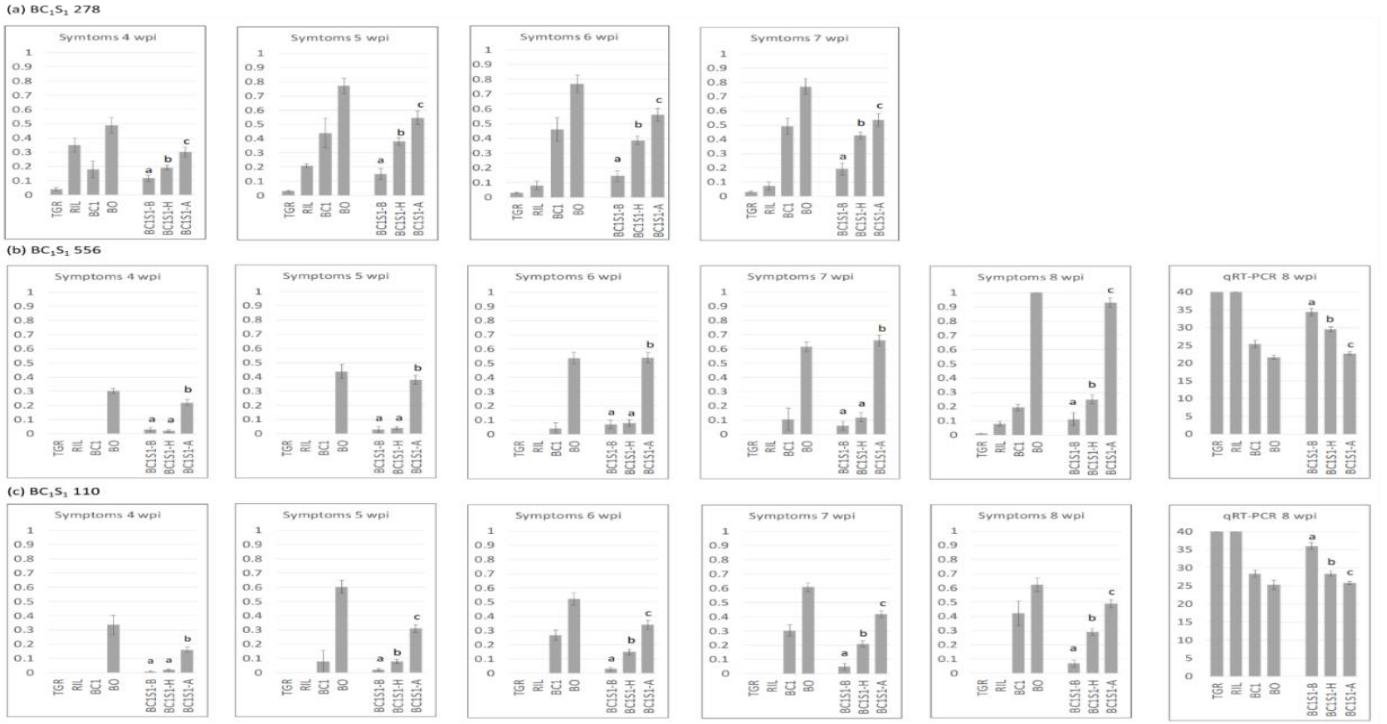


Figure 2. (a) $BC_1S_1$278; (b) $BC_1S_1$556 (c) $BC_1S_1$110. Average symptom score (percentage of leaves showing clear symptoms of CYSDV infection) for different evaluation dates (wpi: weeks post infection) and average Ct (cycle threshold) in the qRT-PCR carried out to detect CYSDV (when available). Genotypes are expressed as: TGR: TGR-1551; RIL: selected RIL, RIL278, 556 or 110; BC_1: first backcross to 'Bola de Oro' of the selected RIL; BO: 'Bola de Oro'; BC_1S_1: selfing progeny from the BC_1, classified according to the genotype for marker cysdv22 for RIL278 and marker cysdv63 for RILs 556 and 110, BC_1S_1-B (homozygous for TGR-1551 allele), BC_1S_1-H (heterozygous) and BC_1S_1-A (homozygous for 'Bola de Oro' allele). Bars represent standard error. Different letters in the same evaluation date indicate significant differences among genotypes ($p<0,05$, LSD test).

For subsequent analyses, the availability of new GBS data (GBS2) allowed the design of molecular markers in the 10 cM region lacking markers in GBS1 (**Figure 1**) to better delimit the candidate interval. This new set of markers (CYSDV2) was used to genotype the 134 $BC_1S_1$556 plants, as evaluation period in this population extended to 8 wpi, when virus titer was measured in all plants. A new map of the region was constructed with the genotyping data of the plants with both markers set, CYSDV1 and CYSDV2, which covered 62.5 cM, corresponding to the region from 6,412,266 bp to 27,806,568 bp (**Figure 1**) to perform the QTL analysis of this population more accurately. The new QTL analyses, using this map and the $BC_1S_1$556 population phenotyping results, confirmed the occurrence of a major QTL in this region of chromosome 5 (**Table 3**). The intervals for the QTL considering the different evaluation dates were overlapping, and altogether covered the region between 25,619,503 and 26,688,074 bp. The closest marker to the LOD peak was *cysdv63*. Almost the same interval (25,619,503-26,629,653 bp) corresponded to the QTL obtained when assigning a phenotype to each RIL, considering the symptoms evaluation and the virus titer (**Table 3**). In any case, the percentage of the variance explained for the different evaluation dates showed an increasing trend with time (from 33% to 56%). Evaluation 5 (8 wpi) was the date that explained the highest percentage of the variation, along with the highest LOD. The interval for the QTL in this evaluation date comprised the region between 25,982,529 and 26,629,653 bp, thus suggesting that this late phenotyping allowed a more accurate characterization of the population. A second QTL associated to the qRT-PCR was identified in the interval between markers *cysdv17* and *cysdv18* (22,651,076-24,296,585 bp), which explained 44% of the variation for the trait (**Table 3**).

To evaluate the effect of the symptoms QTL in both progenies, $BC_1S_1$278 and $BC_1S_1$556 plants were grouped according to their genotype for the closest marker to the higher peak available in each case (*cysdv22* for $BC_1S_1$278 and *cysdv63* for $BC_1S_1$556). The average symptom score in each evaluation dates was calculated for each genotypic class (**Figure 2**). In the case of $BC_1S_1$556 virus titer was also available. Symptom scores were significantly higher in homozygotes for BO allele with respect to the rest of the genotypes in BC_1S_1, from the first evaluation dates. Symptoms in homozygous plants for the BO allele were slightly lower than in BO, corresponding to a slightly lower virus titer (evaluated in $BC_1S_1$556). Differences between heterozygous BC_1S_1 plants and homozygous for the TGR allele were significant for all evaluation dates in $BC_1S_1$278, but only at 8 wpi in $BC_1S_1$556. In the case of heterozygotes BC_1S_1 plants, the average symptom score was similar to those in the corresponding BC_1, while virus titer (measured in the 556 progenies) was lower in the former. Average scores in homozygotes for TGR allele were higher than those

observed in the resistant parent, TGR, and in the corresponding RILs (only in the final evaluation date in the case of $BC_1S_1$278), given that as previously explained, the observed yellowing in the RILs were not attributable to the presence of virus.

To confirm the previous results, BC_1S_1 progeny derived from RIL110 was phenotyped for resistance to CYSDV in 2017 (**Supplementary Table S1**). Percentage of plant infection did not reach 100% in the susceptible control BO at the end of the assay (8 wpi), remaining at levels similar to those obtained in the $BC_1S_1$278 assay, thus suggesting lower inoculum pressure with respect to the $BC_1S_1$556 assay (**Figure 2**). Similarly, the virus titer detected in the susceptible control in $BC_1S_1$110 assay was significantly lower at 8 wpi than in $BC_1S_1$556 assay. Resistant controls displayed similar response in the three assays as TGR-1551 and the resistant RILs remained virus free. On the contrary, symptom scores in $BC_1$110 and $BC_1$278 were higher than in $BC_1$556, while virus titer was comparable in both assays.

The 146 $BC_1S_1$110 plants were classified according to their genotype for marker *cysdv63* (**Figure 2**). Differences between the three genotypic classes were significant from the second evaluation date to the end of the assay. Symptoms in BC_1S_1 plants homozygotes for BO allele were lower than in the assay with family 556, supporting the results obtained in the susceptible control BO. In any case, symptoms in homozygotes BC_1S_1 plants for the BO allele were slightly lower than in the susceptible parent, BO. Similarly, symptoms in BC_1S_1 heterozygous for *cysdv63* were lower than in the heterozygous BC_1 for most evaluation dates. Homozygotes for the TGR allele showed slight symptoms, corresponding to the detection of virus titer, while TGR and RIL110 remained asymptomatic and virus free.

Differences found among the three BC_1S_1 populations (all segregating for the TGR-1551 candidate introgression in chromosome 5) could be explained by differences in the genetic background for the rest of the genome. Moreover, as previously stated, there is an important effect of environmental conditions on TGR-1551-derived resistance to CYSDV (McCreight *et al.*, 2019). The most important differences in this work were found in heterozygous genotypes. These results agree with differences found in previous works, where the F_1 generation derived from the initial cross TGR-155 x BO behaved either as resistant (López-Sesé & Gómez-Guillamón, 2000) or susceptible (McCreight *et al.*, 2019). The heterozygous plants in the three segregating BC_1S_1 populations analyzed here showed (for the percentage of infection in the later evaluation dates, 8wpi) an intermediate response between TGR-1551 and BO. However, the evolution in these heterozygous plants from the segregating generations supported the previous findings that established later evaluation dates as more representative (McCreight

et al., 2019), as in some of the assays percentage of infection in the initial sampling dates did not differ from that of the homozygotes for the TGR-1551 allele.

Table 3. Quantitative trait loci (QTLs) identified in chromosome 5 in BC_1S_1 progenie of RIL 556 derived from the cross between TGR-1551 and the cultivar 'Bola de Oro', phenotyped for resistance to cucurbit yellow stunting disorder virus and genotyped with markers set CYSDV1 and CYSDV2 (see text for description).

Trait [1]	Interval [2]	Nearest marker[3]	Composite interval mapping	
			LOD [4]	R^2 [5]
S4wpi	44.0-50.5 cM 25,619,503-26,688,074 bp	*cysdv63*	14.8	0.33
S5wpi	45.7-49.9 cM 25,982,529-26,629,653 bp	*cysdv63*	19.9	0.39
S6wpi	45.8-49.9 cM 25,982,529-26,629,653 bp	*cysdv63*	19.6	0.39
S7wpi	45.4-49.8 cM 25,943,991-26,629,653 bp	*cysdv63*	17.6	0.39
S8wpi	46.4-49.5 cM 25,982,529-26,629,653 bp	*cysdv63*	33.1	0.56
qRT-PCR	20.8-24.8 cM 22,651,076-24,296,585 bp	*cysdv17*	20.4	0.44
Response	44.2-50.1 cM 25,619,503-26,629,653 bp	*cysdv63*	16.0	0.26

[1] Trait: Symptom: evaluation in different dates, indicated as S (number of weeks) wpi (wpi: weeks post inoculation). qRT-PCR: Ct value for the qRT-PCR at 8wpi. Response: classification of each plant as resistant, moderately resistant, moderately susceptible or susceptible considering symptom evaluation and qRT-PCR Ct value. [2] Interval position of the putative QTL on the genetic and the physical map according to a LOD drop of 2. The physical position (v.3.6.1) is defined by the position of the markers flanking the QTL interval. [3] Closest marker to the LOD peak. Significance level in the Kruskal-Wallis test was 0.0001 for all the markers. [4] Higher logarithm of the odds score. [5] Percentage of phenotypic variance explained by the QTL.

2.4. Progeny test narrowing the interval of the major QTL and confirmation of its effect in advanced backcross selfing populations

To complement the analysis of the RILs and BC_1S_1 RILs derived populations, and to evaluated the effect of the introgression in plants with BO genetic background, 200 plants of the BC_3 (TGR x BO derived) population were genotyped with markers set CYSDV1. Fifty-two plants were selected for the presence of different introgressions in the candidate region of chromosome 5. These plants were additionally genotyped with a set of 124 markers evenly distributed throughout the genome. As an average, 82% of the genome in the 52 plants corresponded to regions homozygous for BO alleles. Fifteen BC_3 plants were selected to obtain the BC_3S_1 progenies. The selection was based on the presence of different introgressions in the candidate region of chromosome 5 (**Table 4**), and prioritizing a high percentage of the BO genetic background for the rest of the genome.

The phenotyping/genotyping results of these offsprings were compatible with the interval for the major QTL obtained in the analyses of the $BC_1S_1$556 plants (**Table 4, Supplementary Table S1**). Recombinants in the region allowed the delimitation of the candidate region. The interval obtained considering the different evaluation dates in $BC_1S_1$556 plants covered the region between 25,619,503 and 26,688,074 bp. Segregation among BC_3S_1 plants derived from BC_3 166 and 198, and susceptibility of descendants from BC_3 105, 141 and 146, confirmed the location of the resistance gene between markers *cysdv63* and *cysdv65* (25,982,529-26,629,653 bp). All BC_3 progenies segregated according to the presence of the resistance gene/s in the region between markers *cysdv63*-*cysdv65*, producing susceptible selfing progenies (24, 28, 37, 78 and 96, 105, 141 y 146), or segregating offsprings (15, 19, 64, 95, 159, 166 and 198) (**Table 4**). The proposed interval explained the phenotype of the RILs; excluding recombinants in the region, for the rest of the RILs there was cosegregation between the phenotype and the genotype in 87% of the homozygous lines (**Supplementary Table S1**).

Table 4. Genotype for SNPs in marker set CYSDV1 and CYSDV2, for the BC_3 plants selected to evaluate their descendants (A: homozygous for 'Bola de Oro' allele; H: heterozygous). The phenotype of the progenies is indicated (SU: susceptible; SE: segregating)

Marker	Position (bp)	15	19	64	95	159	166	198	24	28	37	78	96	105	141
cysdv10B	6,412,266	A	A	H	A	H	H	A	H	H	A	A	A	A	A
cysdv11	9,593,263	A	A	H	A	H	H	A	H	H	A	A	A	A	A
cysdv14	17,265,147	A	A	H	A	H	H	A	H	H	A	A	A	A	A
cysdv17	22,651,076	A	A	H	A	H	H	A	H	H	A	A	A	A	A
cysdv18	24,296,585	A	H	H	A	H	H	A	A	H	A	A	A	A	A
cysdv19	24,365,016	A	H	H	A	H	H	A	A	H	A	A	A	A	A
cysdv21	24,605,826	A	H	H	A	H	H	A	A	H	A	A	A	A	H
cysdv22	24,613,028	A	H	H	A	H	H	A	A	H	A	A	A	A	H
cysdv40	24,652,307	A	H	H	A	H	H	A	A	H	A	A	A	A	H
cysdv42	24,792,185	A	H	H	A	H	H	A	A	A	A	A	A	A	H
cysdv43	24,864,545	A	H	H	A	H	H	A	A	A	A	A	A	A	H
cysdv44	24,890,589	A	H	H	A	H	H	A	A	A	A	A	A	A	H
cysdv45	24,945,626	A	H	H	A	H	H	A	A	A	A	A	A	A	H
cysdv46	24,962,187	A	H	H	A	H	H	A	A	A	A	A	A	A	H
cysdv48	25,026,788	A	H	H	A	H	H	A	A	A	A	A	A	A	H
cysdv49	25,027,045	A	H	H	A	H	H	A	A	A	A	A	A	A	H
cysdv50	25,236,105	H	H	H	A	H	H	A	A	A	A	A	A	A	H
cysdv51	25,314,484	H	H	H	H	H	H	A	A	A	A	A	A	A	H
cysdv53	25,326,351	H	H	H	H	H	H	A	A	A	A	A	A	A	H
cysdv54	25,392,541	H	H	H	H	H	H	A	A	A	A	A	A	A	H
cysdv55	25,392,903	H	H	H	H	H	H	A	A	A	A	A	A	A	H
cysdv56	25,415,551	H	H	H	H	H	H	A	A	A	A	A	A	A	H
cysdv57	25,526,168	H	H	H	H	H	H	A	A	A	A	A	A	A	H
cysdv58	25,540,372	H	H	H	H	H	H	A	A	A	A	A	A	A	H
cysdv59	25,619,503	H	H	H	H	H	H	A	A	A	A	A	A	A	H
cysdv60	25,943,991	H	H	H	H	H	H	A	A	A	A	A	A	A	H
cysdv61	25,956,650	H	H	H	H	H	H	A	A	A	A	A	A	A	H
cysdv62	25,975,889	H	H	H	H	H	H	A	A	A	A	A	A	A	H
cysdv63	25,982,529	H	H	H	H	H	H	A	A	A	A	A	A	A	H
cysdv65	26,629,653	H	H	H	H	H	A	H	A	A	A	A	A	H	A
cysdv69	26,688,074	H	H	A	H	H	A	H	A	A	A	A	A	H	A
cysdv24	26,766,636	H	H	A	H	H	A	H	A	A	A	A	A	H	A
cysdv23	26,976,581	H	H	A	H	H	A	H	A	A	A	A	A	H	A
cysdv25	26,993,475	H	H	A	H	H	A	H	A	A	A	A	A	H	A
cysdv26	27,170,637	H	H	A	H	H	A	H	A	A	A	A	A	H	A
cysdv27	27,188,971	H	H	A	H	H	A	H	A	A	A	A	A	H	A
cysdv28	27,353,196	H	H	A	H	H	A	H	A	A	A	A	A	H	A
cysdv29	27,772,725	H	H	A	H	H	A	A	A	A	A	A	A	H	A
cysdv30B	27,806,568	H	H	A	H	H	A	H	A	A	A	A	A	H	A
Phenotype		SE	SE	SE	SE	SE	SE	SE	SU	SU	SU	SU	SU	SU	SU

The candidate interval for the CYSDV resistance major QTL obtained in this work (25,982,529-26,629,653 bp) contains 57 predicted genes, 51 of them annotated (**Supplementary Table S5**). Several of the annotated genes in this region have resistance-related functions. In fact, the candidate interval overlaps with a 760 kb region with the highest concentration of resistance genes in the melon genome

(González *et al.*, 2013). This region has been shown to be highly polymorphic at the intra- and interspecific levels, thus explaining differences in resistance found in different melon genotypes (González *et al.*, 2013). The interval proposed here contains three genes annotated as 'disease resistance protein' (MELO3C031332.2, MELO3C004320.2 and MELO3C031556.2), as well as related-resistance genes such as a receptor-like cytosolic serine/threonine-protein kinase (MELO3C004315.2) or two nucleotide-binding site-leucine rich repeat proteins (MELO3C004319.2, MELO3C031325.2). The most frequent class was the TMV resistance protein N-like (12 of the annotated genes in the candidate interval), a TIR-NBS-LRR gene having homology with resistance genes (González *et al.*, 2013). Moreover, this region includes the *virus aphid transmission resistance gene* (*Vat*) (González *et al.*, 2013), carried by TGR-1551 (Sarria-Villada *et al.*, 2009). The major QTL for resistance to powdery mildew caused by *Podosphaera xanthii* (Castagne) U. Braun & N. Shishkoff races 1, 2, and 5 derived from the resistance source used here, TGR-1551, has also previously mapped to this region (Yuste-Lisbona *et al.*, 2011). This will be an advantage in the breeding program for the introgression of TGR-1551 resistance to either CYSDV, *Aphis gosypii* or powdery mildew, given that the three resistances would be transferred simultaneously. In fact, the Yellow Canary breeding melon line 'Carmen', derived by backcrossing from the initial cross between TGR-1551 and 'Bola de Oro', confirmed this instance (Palomares-Rius *et al.*, 2018). Although resistance to CYSDV was the only selection character used in the backcrossing program, the line 'Carmen' resulted also highly resistant to powdery mildew and *Aphis gosypii*. Furthermore, quality was assessed in 'Carmen', which confirmed its commercial quality despite carrying the introgression associated with the resistances. TGR-1551 is also resistant to watermelon mosaic virus (WMV) and molecular markers tightly linked to the resistance QTL with major effect on chromosome 11 have been developed (Pérez-de-Castro *et al.*, 2019). Resistance to WMV can also be introgressed into elite cultivar in breeding programs with TGR-1551 as donor parent. Apart from a major QTL of chromosome 11, a minor QTL also associated with resistance to WMV derived from this source is located in this same region of chromosome 5 (Pérez-de-Castro *et al.*, 2019).

Resistance to CYSDV in cucumber (*Cucumis sativus* L.) has been reported in accession PI 250147, characterized by the absence of symptoms (De Ruiter *et al.*, 2008). One of the QTLs, which explained the highest percentage of this resistance, seemed to be linked in the repulsion phase to two *loci* conferring resistance to powdery mildew, derived from different sources. These resistances have been mapped to linkage group 5, in a region syntenic to melon chromosomes 9 and 10. A method to introgress resistance to CYSDV in melon chromosome 9 from an

unknown source has been patented (García-Andrés *et al.,* 2016), but the region is not syntenic with the CYSDV-resistance region in cucumber.

The region of the melon genome interval containing the cluster of putative resistance genes has been studied in detail to analyze the genomic variability (González *et al.*, 2014). Their study confirmed the difficulty in obtaining an accurate and complete sequence by NGS, due to the multiple, highly similar genes clustered in a relatively short region. This may have been the cause of the lack of markers in this region in the initial map developed from the GBS of the RILs population. In any case, the availability of the subsequent GBS data allowed the identification of markers polymorphic between BO and TGR-1551 to design the markers set CYSDV2, which led to the narrowing of the candidate interval for the TGR-1551-derived resistance. Markers in both sets, CYSDV1 and CYSDV2, were initially implemented for their use in the Agena Bioscience genotyping platform. Some of the markers of both sets have been adapted for their analysis by high resolution melting (HRM). Concretely, the HRM protocol for markers in the candidate regions has been set (**Supplementary Table 4**), which allows the efficient identification of the polymorphism between the BO and the TGR allele by PCR-based markers. The availability of this type of markers, suitable for MAS in the context of breeding programs, is of special interest in the case of introgression of resistance to CYSDV. As previously stated, the yellowing symptoms caused by crinivirus are undistinguishable, not only among them (Abrahamian & Abou-Jawdah, 2014), but also from nutritional disorders or phytotoxicity (Wisler *et al.*, 1998). Thus, the use of MAS is an effective method to circumvent these difficulties.

The analysis of different TGR-1551-derived generations allowed the narrowing of the candidate interval of resistance to CYSDV to a 700-kb region in chromosome 5. This resulted in the availability of molecular markers tightly linked to the resistance. The PCR-based markers here developed are an efficient resource for their use in TGR-1551-derived resistance introgression in commercial melons. The breeding program for the introgression of TGR-1551-derived resistance to CYSDV, WMV and powdery mildew in 'Bola de Oro' and 'Piel de Sapo' backgrounds has already been initiated. Future work will include further analysis in advanced backcrossed progenies with the purpose of better understand the effect of the minor QTL obtained in some of the analyses in the region of markers *cysdv14* and *cysdv17*. The linkage between this region and the region of the major QTL limits the availability of segregant generations appropriate to discriminate against the effect of this minor QTL. Moreover, RNA-seq analysis will be carried out in order to identify differentially expressed genes in the candidate regions.

3. Materials and Methods

3.1. Plant material

The following populations obtained from an original cross between the resistant melon line TGR-1551 and the susceptible Spanish melon cultivar 'Bola de Oro' (BO) have been evaluated against CYSDV (**Supplementary Figure S1**):

- A RIL population F_7/F_8, developed by the single seed descent method (Palomares-Ríus *et al.*, 2016)
- Three BC_1S_1 progenies derived from crosses between three resistant RILs and BO
- Fifteen BC_3S_1 progenies derived from 15 BC_3 (x BO) selected for their genotype in the candidate region.

The RIL population (3-4 plants/RIL) together with the parental genotypes and its F_1, was evaluated in five different assays (number of lines in brackets): Spring 2009 (86), Spring 2010 (101), Spring 2011 (73), Summer 2012 (88) and Summer 2013 (121). Not all RILs were evaluated in all the assays due to seed availability. The different backcross-selfing populations were phenotyped in additional screening assays according to the methodology described below.

3.2. Inoculation method

Healthy whitefly colonies (*Bemisia tabaci*) reared on plants of the susceptible Spanish melon accession 'ANC-57' were used in all the inoculations and assays. Controlled CYSDV infected plants of this accession showing clear and typical symptoms of CYSDV were used as inoculum source of the virus in all the inoculations.

RIL assays conducted in 2009 and 2011 were carried out by using *B. tabaci* confined in clip-cages following the methodology developed by (Célix *et al.*, 1996), in which 60 whiteflies are fed in the inoculum source for 48 h (acquisition time) and then transferred to young plants (1-2 true leaf) allowing them to feed on for 48 hours (transmission time). Once inoculated, plants were sprayed with imidacloprid to kill the whiteflies and transplanted to pots in the glasshouse. Assays conducted in 2010, 2012 and 2013 were carried out by massive inoculation. Plants of each RIL at 2-3 true leaves stage were transplanted to plastic greenhouse where they were randomly distributed interspersed with BO infected plants. One week after transplanting viruliferous whiteflies were released in the plot to enhance virus transmission. In this case, any spraying against whiteflies was done over the course of the experiment.

Clip-cage method was used in the inoculation of subsequent generations.

3.3. Disease assessment

Virus symptoms were visually assessed once a week over a month period (2009, 2010 and 2011) or over a two-month period (2012 and 2013). The symptom scoring in Spring 2009 and Spring 2010 was based on a visual scale of the number of leaves with viral symptoms, ranging from 0 = no symptoms to 9 = nine leaves with clear virus symptoms. In Spring 2011 and Summer 2012 the score was based on a visual scale of virus symptoms, ranging from 0 = no symptoms to 5 = almost the entire plant with clear symptoms. In Summer 2013, the scoring was based on the percentage of leaves showing typical CYSDV symptoms of infection (number of leaves affected/total leaf number).

In the trials of 2012 and 2013, the third leaf from the plant apex of each plant was sampled fourteen days after virus inoculation (2012) or each week (2013) to estimate the virus titer in the plants by qRT-PCR following (Gil-Salas *et al.*, 2007).

During the Spring 2016 and Spring 2017 three BC_1S_1 progenies derived from crosses between three resistant RILs (278, 556 and 110) and BO were evaluated. Virus inoculations were carried out by using *B. tabaci* confined in clip-cages as explained above. Once inoculated, plants were transplanted to the greenhouse. Evaluation against CYSDV was based on the percentage of leaves showing typical CYSDV symptoms of infection (number of leaves affected/total leaf number). qRT-PCR analyses to estimate the virus titer in the plants were carried out every week (2016) or every two weeks (2017).

Additionally, in 2019 BC_3S_1 progenies (twelve to twenty plants each) derived from 15 BC_3 plants selected according to their genotype in the candidate region of chromosome 5 were inoculated with CYSDV using the clip-cage method. Then, plants were transferred to the greenhouse and evaluated by symptoms and qRT-PCR as described for the RIL110BC_1S_1 population.

3.4. Statistical analyses

Analysis of variance (ANOVA) using data from four of the RIL evaluations was carried out. Correlations between pairs of environments were estimated by using the Pearson correlation coefficient. Comparisons of pairs of means in different assays were performed using the LSD test with a probability level of $p<0.05$. Statistical analyses were conducted using Statgraphics Centurion XVI.I software.

3.5. Markers and genotyping methods

Total DNA was extracted from young leaves following the method described by (Doyle & Doyle, 1990) with minor modifications (Esteras *et al.*, 2013). DNA concentration was measured using spectrophotometry in a Nanodrop ND-1000 Spectophotometer v.3.5 and adjusted to 10 ng/□L for the genotyping analysis.

The SNPs used in this study were designed from data originated in different GBS analysis. The whole RIL population (148 RILs), both parents (BO and TGR-1551) and their F1, constituted the plant materials for the first GBS (GBS1 assay). A total of 16 SNPs, located on the candidate region on chromosome 5, were selected from this GBS and constituted the first set of markers (CYSDV1, **Supplementary Table S4**). Preferably, markers were selected that correspond to those included in the map used in the analysis. When necessary to evenly cover the region, markers were chosen among the markers discarded for the map because of segregation distortion (only two markers). An additional GBS experiment, GBS2, conducted to perform genetic diversity studies (including many genotypes, among others, BO and TGR-1551), were also used as a source for 24 extra SNPs, to increase resolution on the candidate region (markers set CYSDV2, **Supplementary Table S4**). All the SNPs were implemented for their use in the Agena Bioscience platform, carried out in the "Epigenetic and Genotyping unit of the University of Valencia, Unitat Central d´Investigació en Medicina (UCIM), Spain". Markers set CYSDV1 was used to genotype BC_1S_1, BC_3 and BC_3S_1 generations. Generations $BC_1S_1$556 and BC_3S_1 were also genotyped with markers set CYSDV2. BC_3 plants were genotyped with an existing panel of 124 SNPs evenly distributed throughout the genome, also implemented in the Agena Bioscience platform. This SNP set had been previously validated in populations derived from ibericus x acidulus melon crosses (Esteras *et al.*, 2013; Leida *et al.*, 2015; Perpiñá *et al.*, 2016). Some of the markers in CYSDV1 and CYSDV2 sets have been adapted to a PCR-based protocol for their analysis by high resolution melting (HRM) (**Supplementary Table S4**).

3.6. QTL analyses

QTL analysis was performed using the genotyping results of GBS1 and RILs CYSDV phenotype. Phenotypic data used were the percentage of infection at the end of each assay and qRT-PCR values, when available. Moreover, a final phenotypic value was assigned to each RIL, considering the five evaluations, so that each RIL was classified as resistant or susceptible. First, Kruskal-Wallis non-parametric test was used for QTL detection (MapQTL version 4.1 software) (Van Ooijen, 2009). In addition, a composite interval mapping (CIM) approach was performed (Zeng,

1994), using a windows size of 15 cM and five cofactors (Windows QTL Cartographer v.2.5-009) (Wang, 2012). For qualitative traits (response, where plants were classified as either susceptible or resistant), qGene v.4.4.0 was the software used (Joehanes & Nelson, 2008). The LOD threshold was determined by a permutation test (1,000 cycles). *Loci* detected by both, Kruskal-Wallis and CIM methods, were considered robust QTLs. QTL interval was defined as a 2-LOD drop from the peak. The phenotypic effect, expressed as the percentage of phenotypic variance explained, R^2, and the additive (when possible) and dominance effects were estimated for each QTL. The map used in the QTLs analyses for the RIL population was that constructed with the GBS data of the RILs (Pérez-de-Castro *et al.*, 2019).

Additional QTL analyses were performed with BC_1S_1 progenies from RILs 278 and 556. BC_1S_1 progenies from RIL 278 were genotyped with a set of SNP markers selected from the GBS of the RILs (CYSDV1), covering the candidate region in chromosome 5. A new set of markers (CYSDV2) was designed, which saturated the candidate region, and was used, together with CYSDV1, to genotype the BC_1S_1 progenies from RIL 556. A new map of the region was constructed for each of the progenies and used, respectively, for each QTLs analysis. The software used was MAPMAKER 3.0 (Lincoln, 1993). The map was generated using the Kosambi map function.

4. Conclusions

No commercial varieties resistant to CYSDV are available to date. The African accession TGR-1551 has been reported as resistant to *Bemisia tabaci* and to the virus itself. The work reported has allowed the narrowing of the candidate interval for the major QTL associated with resistance to CYSDV derived from TGR-1551 to a region of approximately 700 kb. The SNP markers here provided are useful in marker assisted selection (MAS) in breeding programs aimed at the introgression of CYSDV resistance. CYSDV is exclusively transmitted by its insect vector, *Bemisia tabaci*. Moreover, yellowing symptoms caused by CYSDV are difficult to distinguish from symptoms caused by other viruses, and yellowing provoked by crinivirus are easily confused with nutritional disorders of phytotoxicity, among others. Thus, the availability of markers for MAS in the context of melon breeding programs is essential to accelerate the introgression of resistance to CYSDV into elite cultivars.

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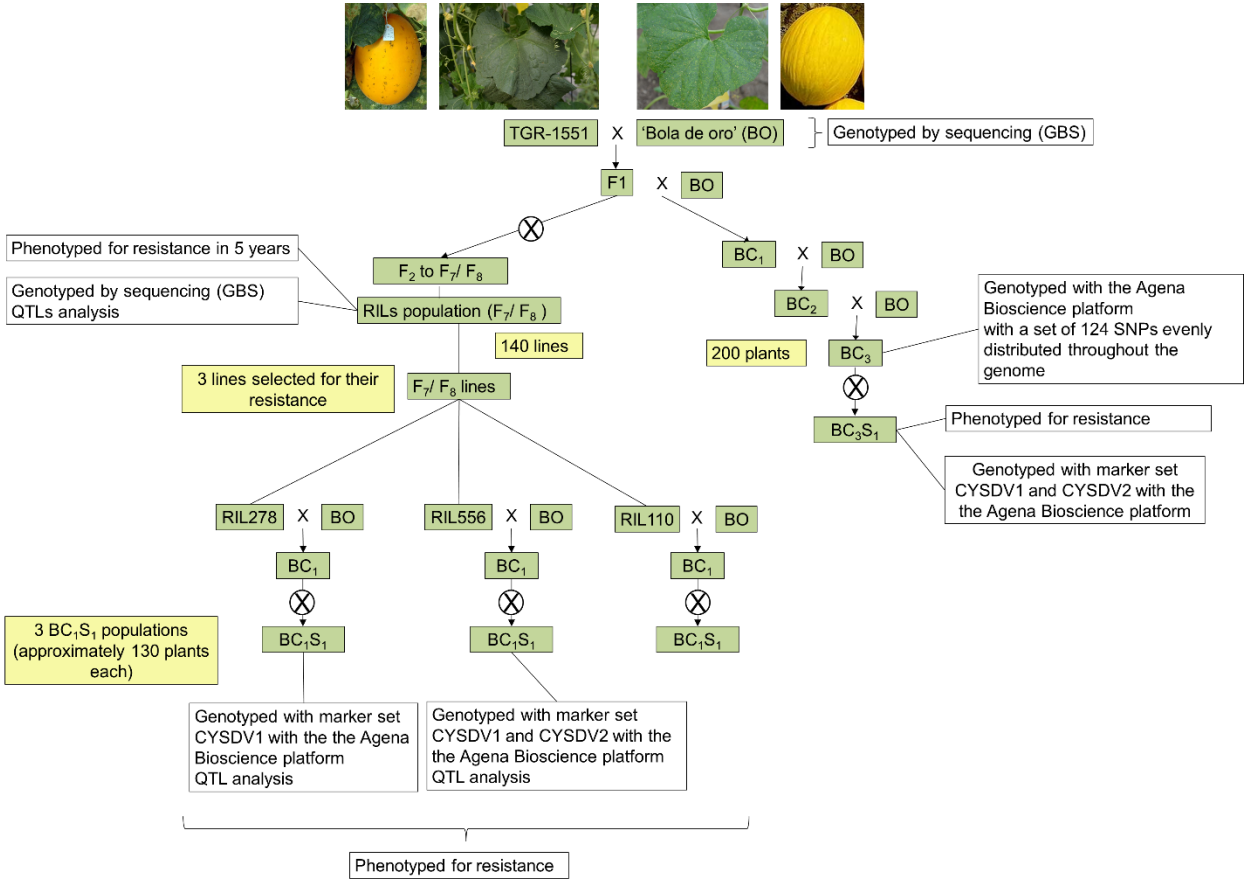
Supplementary Materials

Supplementary materials can be found at **https://www.mdpi.com/1422-0067/21/17/5970/s1**

Figure S1. Generations used and experiments carried out.



Supplementary Table S1. Symptom evaluation and qRT-PCR result (positive/negative or Ct value) after inoculation with CYSDV of different populations, a RIL population and advanced derived backcrosses. Each sheet gathers data from one of the populations studied.

Due to the extent of this supplementary material is available at https://www.mdpi.com/1422-0067/21/17/5970/s1

Supplementary Table S2. Correlations (and significance values, below, highlighted in grey) between parameters used for disease assessment (symptom scores and qRT-PCR Ct values in the RILs population in the different assays, wpi: weeks post infection).

	symptoms 2009	symptoms 2010	symptoms 2011	symptoms 2011	symptoms 2013	qRT-PCR 2012	qRT-PCR 2013 (5 wpi)	qRT-PCR 2013 (8 wpi)
symptoms 2009		0.463	0.448	0.539	0.367			
		0.0001	0.0002	0.0001	0.0018			
symptoms 2010			0.839	0.663	0.548			
			0.0001	0.0001	0.0001			
symptoms 2011				0.619	0.709			
symptoms				0.0001	0.0001			
symptoms 2012					0.664	0.776		
					0.0001	0.0001		
symptoms 2013							0.699	0.513
							0.0001	0.0001
qRT-PCR 2012							0.824	0.823
							0.0001	0.0001
qRT-PCR 2013 (5 wpi)								0.750
								0.0001
qRT-PCR 2013 (8 wpi)								

Supplementary Table S3. Genotype of RILs 110, 278 and 556 for the markers in GBS 1 (A: homozygous for 'Bola de Oro' allele; B: homozygous for TGR-1551 allele; H: heterozygous). The name of the markers indicates the linkage group and the physical position (bp) in version v3.6.1 of the melon genome (available at https://www.melonomics.net/). Markers in the QTL intervals obtained with the RILs population are highlighted in grey.

Available at https://www.mdpi.com/1422-0067/21/17/5970/s1

Supplementary Table S4. Marker sets (CYSDV1 and CYSDV2) used in genotyping of progenies. The SNPs were obtained from two different genotyping experiments, GBS1 and GBS2 (see text for description). The physical position in version v.3.6.1. of the melon genome (CM3.6.1, available at https://www.melonomics.net/) is indicated. The SNP (in brackets) and flanking sequences are included. Next sheet includes the HRM protocol for selected markers.

Available at https://www.mdpi.com/1422-0067/21/17/5970/s1

Supplementary Table S5. Gene loci in the region of the candidate interval for the major QTL for resistance to CYSDV derived from TGR-1551, according to the last version of the genome annotation (v.4, available at https://www.melonomics.net/).

Gene locus	Locus start (pb, v3.6.1)	Locus end (pb, v3.6.1)	Strand	Description
MELO3C004284.2	25980674	25984043	+	Increased DNA methylation 2
MELO3C004285.2	25984965	25988828	-	signal peptidase complex subunit 3B
MELO3C004286.2	25994809	25996153	-	E3 ubiquitin-protein ligase ATL6
MELO3C004287.2	26033818	26036127	+	transcription factor bHLH94-like
MELO3C004288.2	26044574	26052361	+	TMV resistance protein N-like
MELO3C004289.2	26065157	26071880	-	TMV resistance protein N-like
MELO3C004291.2	26106290	26108583	+	TMV resistance protein N-like
MELO3C004292.2	26120489	26126516	+	TMV resistance protein N-like isoform X1
MELO3C004294.2	26150345	26154949	+	TMV resistance protein N-like isoform X1
MELO3C000887.2	26185828	26186458	+	Kunitz-type trypsin inhibitor
MELO3C004296.2	26189506	26190684	+	endogenous alpha-amylase/subtilisin inhibitor-like
MELO3C004297.2	26193895	26210413	-	Branched-chain-amino-acid aminotransferase-like protein
MELO3C031550.2	26197200	26197379	+	Unknown protein
MELO3C004298.2	26210218	26210410	+	Unknown protein
MELO3C004299.2	26216801	26220705	+	GDSL esterase/lipase At1g54790-like
MELO3C004300.2	26223696	26229871	+	GDSL esterase/lipase
MELO3C004301.2	26231021	26237770	-	TMV resistance protein N-like isoform X1
MELO3C004303.2	26239395	26244020	-	TMV resistance protein N-like
MELO3C004302.2	26239400	26240028	-	TMV resistance protein N-like isoform X1
MELO3C031552.2	26243386	26244297	-	TMV resistance protein N-like
MELO3C004305.2	26245451	26251493	+	Pre-mRNA-splicing factor SLU7
MELO3C004306.2	26252325	26255675	+	GDSL esterase/lipase At1g54790
MELO3C004307.2	26255104	26257981	-	UPF0603 protein, chloroplastic
MELO3C004308.2	26259013	26262927	+	Photosystem II reaction center PsbP family protein
MELO3C004309.2	26263738	26270641	+	LOW QUALITY PROTEIN: TMV resistance protein N-like
MELO3C031553.2	26273879	26276251	-	Ulp1 protease family, carboxy-terminal domain protein
MELO3C031551.2	26275108	26275419	+	INE RNAaseH
MELO3C004311.2	26280801	26299171	-	TMV resistance protein N-like
MELO3C004313.2	26311869	26315091	-	TMV resistance protein N-like
MELO3C004314.2	26325323	26326536	+	FANTASTIC four-like protein (DUF3049)
MELO3C004315.2	26339876	26344018	+	receptor-like cytosolic serine/threonine-protein kinase RBK2
MELO3C004316.2	26345715	26347916	+	Prefoldin chaperone subunit family protein, putative
MELO3C004317.2	26351673	26353371	+	Vat protein
MELO3C004318.2	26377701	26379528	+	Vat protein
MELO3C004319.2	26410035	26411751	+	Nucleotide binding site-leucine rich repeat protein
MELO3C031332.2	26410133	26411047	+	Disease resistance protein
MELO3C031554.2	26412341	26413394	+	Vat protein
MELO3C031326.2	26414297	26415620	+	Vat protein
MELO3C031555.2	26415970	26418230	+	Unknown protein
MELO3C004320.2	26423627	26425625	+	Disease resistance protein
MELO3C004321.2	26446783	26457878	+	Vat protein
MELO3C031329.2	26466825	26467911	-	Unknown protein
MELO3C004323.2	26485770	26486695	+	Vat protein
MELO3C031325.2	26486568	26487010	+	Nucleotide binding site-leucine rich repeat protein
MELO3C031556.2	26487174	26489292	+	Disease resistance protein
MELO3C031324.2	26498118	26508551	+	Vat protein
MELO3C004329.2	26521943	26570533	+	Terpene cyclase/mutase family member
MELO3C031557.2	26547187	26547561	-	Reverse transcriptase
MELO3C031327.2	26572524	26574018	+	GPI transamidase component Gpi16 subunit
MELO3C031330.2	26582462	26597336	-	Vat protein
MELO3C031328.2	26599536	26599701	+	Unknown protein
MELO3C031331.2	26600570	26600741	-	Terpene cyclase/mutase family member
MELO3C004332.2	26606841	26607023	-	Unknown protein
MELO3C004333.2	26608671	26611538	-	DCD (Development and Cell Death) domain protein
MELO3C004334.2	26615350	26623396	-	RAD-associated E3 ubiquitin-protein ligase HRD1B-like
MELO3C004335.2	26625456	26628372	-	50S ribosomal protein L18
MELO3C004336.2	26629107	26644600	-	Cation-transporting ATPase

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Chapter 3.

Insights into the early transcriptomic response against watermelon mosaic virus in melon

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Abstract

Background

Watermelon mosaic virus (WMV) is one of the most prevalent viruses affecting melon worldwide. Recessive resistance to WMV in melon has previously been reported in the African accession TGR-1551. Moreover, the genomic regions associated to the resistance have also been described. Nevertheless, the transcriptomic response that might infer the resistance to this potyvirus has not been explored.

Results

We have performed a comparative transcriptomic analysis using mock and WMV-inoculated plants of the susceptible cultivar `Bola de oro´ (BO) and a resistant RIL (Recombinant inbred line) derived from the initial cross between TGR-1551 and BO. In total, 616 genes were identified as differentially expressed and the weighted gene co-expression network analysis (WGCNA) detected 19 gene clusters (GCs), of which 7 were differentially expressed for the genotype x treatment interaction term. SNPs with a predicted high impact on the protein function were detected within the coding regions of most of the detected DEGs. Moreover, 3 and 16 DEGs were detected within the QTL regions previously described in chromosomes 11 and 5, respectively. In addition to these two specific genomic regions, we also observe large transcriptomic changes from genes spread across the genome in the resistant plants in response to the virus infection. This early response against WMV implied genes involved in plant-pathogen interaction, plant hormone signal transduction, the MAPK signaling pathway or ubiquitin mediated proteolysis, in detriment to the photosynthetic and basal metabolites pathways. Moreover, the gen MELO3C021395, which coded a mediator of RNA polymerase II transcription subunit 33A (MED33A), has been proposed as the candidate gen located on chromosome 11 conferring resistance to WMV.

Conclusions

The comparative transcriptomic analysis presented here showed that, even though the resistance to WMV in TGR-1551 has a recessive nature, it triggers an active defense response at a transcriptomic level, which involves broad-spectrum resistance mechanisms. Thus, this study represents a step forward on our understanding of the mechanisms underlaying WMV resistance in melon. In addition, it sheds light into a broader topic on the mechanisms of recessive resistances.

1. Introduction

Melon (*Cucumis melo* L.) is one of the main cucurbit crops cultivated worldwide. Its selection in different countries of the Mediterranean basin and Eastern Asia has led to a great phenotypic and genotypic variability (Pitrat, 2016). This diversity is commonly used as a source of alleles in breeding programs to introgress different characters of interest in elite cultivars. Among these programs, those aimed to produce virus-resistant varieties are of particular importance. Viruses in the main melon producing areas have a great yield-limiting potential and are a major economic concern for growers (Velasco *et al.*, 2020). Different studies carried out recently in Europe (Bertin *et al.*, 2020; Desbiez *et al.*, 2020; De Moya-Ruiz *et al.*, 2021), Asia (Nematollahi *et al.*, 2021; Wang *et al.*, 2017a, 2019) and America (Nematollahi *et al.*, 2021; Pozzi *et al.*, 2020) indicate that the potyvirus watermelon mosaic virus (WMV) is the most prevalent virus in cucurbits fields. WMV infection in melon leads to a severe symptomatology, that includes chlorosis, mosaic, leaf distortion, lead tip stunting, as well as the stop of plant growth, which results in yield reduction and fruit quality loss. Moreover, phylogenetic studies have shown that WMV is constantly evolving due to recombination and mutation events, leading to more virulent strains (Desbiez *et al.*, 2020; Khanal *et al.*, 2021; Verma *et al.*, 2020).

Since potyviruses are transmitted in a non-persistent manner, cultural practices and the use of pesticides are useless to control this virus. Several melon accessions have been described as tolerant to WMV (Moyer *et al.*, 1985; Munger, 1991; Provvidenti *et al.*, 1978; Webb, 1967), but only two accessions, PI 414723 and TGR-1551, have been found to be resistant to WMV (Díaz *et al.*, 2003; Provvidenti *et al.*, 1978). The phenotyping and genotyping by sequencing (GBS) of a RIL (Recombinant inbred lines) population derived from the cross between TGR-1551 and the susceptible cultivar `Bola de Oro´ (BO) allowed to map a major resistance QTL, named *wmv*^{1551}, on a 130 kb interval in chromosome 11. Moreover, a minor QTL with a significant effect on heterozygous plants for the introgression in chromosome 11 was also mapped to a 700 kb region on chromosome 5 (Pérez-de-Castro *et al.*, 2019).

Functional characterization of the genes located in the candidate resistance regions is required to understand the molecular resistance mechanisms against WMV in melon. Usually, recessive resistances against viruses are caused by the loss or the mutation of a susceptibility factor, necessary for the virus to complete its life cycle (Fraser, 1990). Actually, a *Vacuolar protein sorting 4* located out of the described candidate region on chromosome 11 was proposed as a susceptibility factor to

WMV infections (Agaoua *et al.*, 2022). Nevertheless, a microarray expression analysis of 17,443 unigenes after the inoculation of TGR-1551 and a susceptible variety with WMV, revealed a great transcriptomic remodeling (Gonzalez-Ibeas *et al.*, 2012). Moreover, the analysis of the small RNAome by high-throughput pyrosequencing after the inoculation with WMV suggests that mechanisms of RNA silencing could also be implied in the resistance (Gonzalez-Ibeas *et al.*, 2011).

The cheapening of new generation sequencing (NGS) technologies has popularized the use of RNA-seq technology to detect differentially expressed genes (DEGs) in cucurbits plants during viral infections (Amoroso *et al.*, 2021; Li *et al.*, 2017; Lou *et al.*, 2020; Sáez *et al.*, 2022; Sun *et al.*, 2017;). Since RNA-seq provides an enhanced transcriptome coverage, with greater accuracy than microarrays, it is more useful to find new transcription features, identify DEGs and alternative gene splicing. Nevertheless, to our knowledge, there are currently no studies using RNA-seq technology to detect differentially expressed genes related to the defense response against WMV. Moreover, to date, as far as we know, there is only one RNA-seq assay has been conducted to understand the resistance mechanisms against viruses in melon (Sáez *et al.*, 2022).

In this study, we provide new insights into the genetic and transcriptomic basis of WMV resistance in melon, by performing and exhaustive comparative transcriptomic analysis between resistant and susceptible melon lines. We used mock-inoculated and WMV infected melon plants of the resistant RIL-10-3, derived from the accession TGR-1551, and its susceptible parental line `Bola de Oro´ to identify the transcriptomic changes implied in the early resistance response to the virus.

2. Results

2. 1 Assessment of RIL-10-3 and BO response to watermelon mosaic virus infection

All the 24 RIL-10-3 plants inoculated with WMV remained symptomless during the assay (**Figure 1a**), while 24/24 inoculated plants of the susceptible cultivar BO started to show mild-severe symptoms (score 2-3) at 10 days post-inoculation (dpi). The symptomatology increased over time and at 15 and 30 dpi severe mosaic and leaf curling was observed in BO plants (**Figure 1a**). Moreover, those plants sampled without symptoms at early infection stages were allowed to regrow and it was possible to identify symptoms in all of them at 30 dpi.

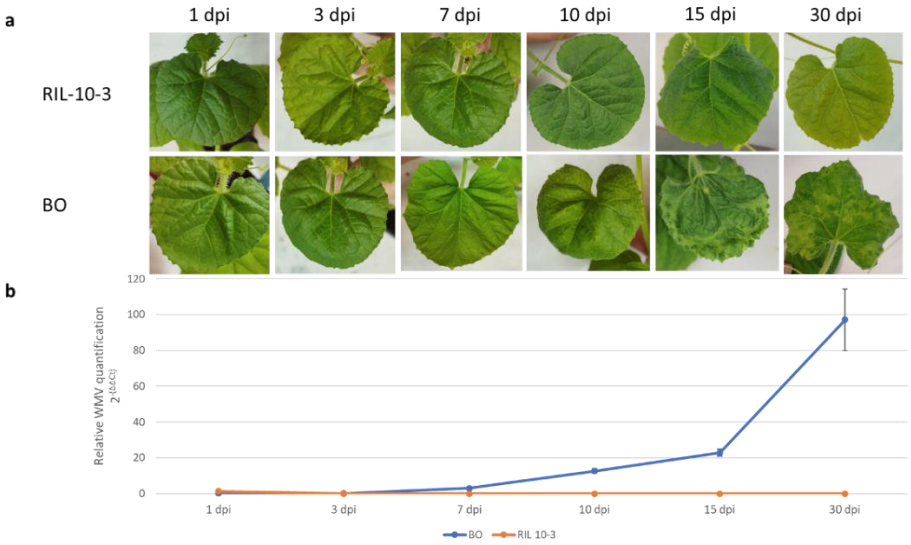


Figure 1. Assesment of RIL-10-3 and BO response to WMV infection in the first total expanded apical leaf of each plant. (a) Temporal evolution of symtomatology. (b) Mean of viral titers in RIL-10-3 and BO measured by RT-qPCR

The accumulation of WMV in both RIL-10-3 and BO inoculated plants was confirmed by RT-qPCR. Significant differences (*p-value*<0.05) were observed between both lines over time. The initial viral accumulation of RIL-10-3 was higher at 1 dpi but it remained at low levels during the course of the experiment, whereas the viral load of BO samples started to rise significantly at 7 dpi, achieving its higher accumulation at 30 dpi (**Figure 1b**). These results show that WMV was able to infect both susceptible and resistant genotypes, but its movement was impaired in the resistant RIL-10-3, whereas in BO the infection was systemic after 7 dpi. These results suggested that the resistance response is activated in the resistant genotype before the infection becomes systemic. Given that at 7dpi the infection was already systemic, we collected samples just in the previous time point, at 3 dpi, to be analyzed by RNA-seq. Moreover, 0 dpi samples were also included as controls.

2.2 RNA sequencing and mapping

A total of 18 libraries were sequenced, producing on average almost 43.7 million reads per sample. After excluding short and low-quality reads, between 20.3 and 29.7 million reads per sample were obtained. The clean reads were mapped to the melon reference genome (v.4.0) (Castanera *et al.*, 2020) with the STAR algorithm (Dobin *et al.*, 2013). The percentage of uniquely mapped reads ranged from 80.65% to 94.62%. (**Table 1**).

Table 1. Summary of the RNA-seq experiment design, number of raw reads, number of clean reads (percentage of raw reads after cleaning), reads uniquely aligned to reference genome and percentage of clean reads mapped (dpi: days post-inoculation).

Sample name	Phenotype	Inoculation treatment	Time point dpi	Replicate	Raw reads	Cleaned reads	Mapped reads	% Uniqueli mapped reads
RIL-10-3-M3dpi_1	Resistant	Mock-inoculated	3	1	41,429,310	20,676,828 (49.91%)	17,808,540	86.16%
RIL-10-3-M3dpi_2	Resistant	Mock-inoculated	3	2	42,112,204	21,017,363 (49.91%)	19,272,192	91.70%
RIL-10-3-M3dpi_3	Resistant	Mock-inoculated	3	3	42,695,048	21,304,504 (49.9%)	17,246,449	80.95%
RIL-10-3-W3dpi_1	Resistant	WMV-inoculated	3	1	41,583,644	20,750,266 (49.9%)	19,102,285	92.06%
RIL-10-3-W3dpi_2	Resistant	WMV-inoculated	3	2	43,083,818	21,508,031 (49.92%)	20,350,015	94.62%
RIL-10-3-W3dpi_3	Resistant	WMV-inoculated	3	3	43,009,278	21,463,096 (49.9%)	19,980,658	93.09%
RIL-10-3-0dpi_1	Resistant	Uninoculated	0	1	40,318,008	20,124,842 (49.92%)	16,545,112	82.21%
RIL-10-3-0dpi_2	Resistant	Uninoculated	0	2	43,093,538	21,504,205 (49.9%)	18,964,458	88.19%
RIL-10-3-0dpi_3	Resistant	Uninoculated	0	3	59,630,926	29,763,672 (49.91%)	24,909,634	83.69%
BO-M3dpi_1	Susceptible	Mock-inoculated	3	1	42,827,098	21,369,863 (49.9%)	17,234,566	80.65%
BO-M3dpi_2	Susceptible	Mock-inoculated	3	2	44,335,964	22,126,097 (49.91%)	20,783,260	93.93%
BO-M3dpi_3	Susceptible	Mock-inoculated	3	3	43,107,228	21,512,325 (49.9%)	19,343,157	89.92%
BO-W3dpi_1	Susceptible	WMV-inoculated	3	1	44,760,600	22,335,730 (49.9%)	20,617,125	92.31%
BO-W3dpi_2	Susceptible	WMV-inoculated	3	2	42,045,224	20,983,927 (49.91%)	18,515,499	88.24%
BO-W3dpi_3	Susceptible	WMV-inoculated	3	3	43,210,794	21,561,209 (49.9%)	18,021,327	83.58%
BO-0dpi_1	Susceptible	Uninoculated	0	1	44,382,722	22,152,146 (49.91%)	19,960,694	90.11%
BO-0dpi_2	Susceptible	Uninoculated	0	2	43,609,702	21,761,448 (49.9%)	20,329,692	93.42%
BO-0dpi_3	Susceptible	Uninoculated	0	3	40,781,540	20,347,309 (49.89%)	18,602,333	91.42%

2.3 Detection of differentially expressed genes and functional classification

The 18 transcriptomes were further analyzed to detect DEGs between the resistant and susceptible genotypes when inoculated with WMV. Two different statistical methods were used (DESeq2 and edgeR) (Robinson *et al.*, 2010; Love *et al.*, 2014). In both cases, genes showing significant differences (significant p-adjusted<0.05) for the interaction term between genotype (resistant or susceptible) and treatment (mock or WMV-inoculated) were considered as DEGs and were divided between down and up-regulated (logFC≤-1 and logFC≥1, respectively). DESeq2 algorithm detected 2,219 DEGs (964 and 2,219 down and up-regulated, respectively) (**Figure 2a)**, while edgeR only detected 657 DEGs (244 and 413 down and up-regulated, respectively) (**Figure 2b**). The consensus list of DEGs between both methods was obtained (**Figure 2c**). As summary, 97.7% of the DEGs detected by edgeR (616 DEGs) were also included in the list of DEGs detected by DESeq2 (**Figure 2d**). Within the down-regulated DEGs, 96.9% of those detected by edgeR were also detected by DESeq2 (213 DEGs) (**Figure 2e**). The proportion was higher within the up-regulated DEGs, as a 99.2% of the DEGs detected by edgeR were also within the DEGs detected by DESeq2 (403 DEGs) (**Figure 2f**). The transcriptomic dataset obtained at 0 dpi was used to check that the observed DEGs were not due to genotype differences. It was checked that none of the DEGs detected between RIL-10-3 and BO at 0 dpi was included among the list of DEGs obtained for the interaction term at 3 dpi. The consensus list of DEGs detected by both edgeR and DESeq2 at 3 dpi was used to further understand the resistance response (**Additional Table 1)**.

DEGs were functionally annotated and GO terms enrichment analysis were performed. On the one hand, the list of up-regulated DEGs was significantly enriched (adjusted p-value<0.05) in “binding” and “helicase activity” molecular functions (**Figure 3a**). This list of genes was also enriched in biological processes such as “regulation of stomatal opening and movement”, “regulation of biological process”, “organelle organization” and “peptidyl-lysine methylation” (**Figure 3c**). On the other hand, for the down-regulated DEGs, non-redundant functions included “ribulose-bisphosphate carboxylase activity”, “electron carrier activity”, “peptidase activity”, “lyase activity”, “catalytic activity”, “DNA helicase activity”, “isomerase activity” and “binding” (**Figure 3b**). The enriched processes were mostly related to “photosynthesis”, “photorespiration”, “DNA replication” and “metabolic processes” (**Figure 3d**). Even though the lists of DEGs were not enriched in “plant-pathogen interaction” or “plant defense” functions, the annotations of several DEGs were related to defense responses.

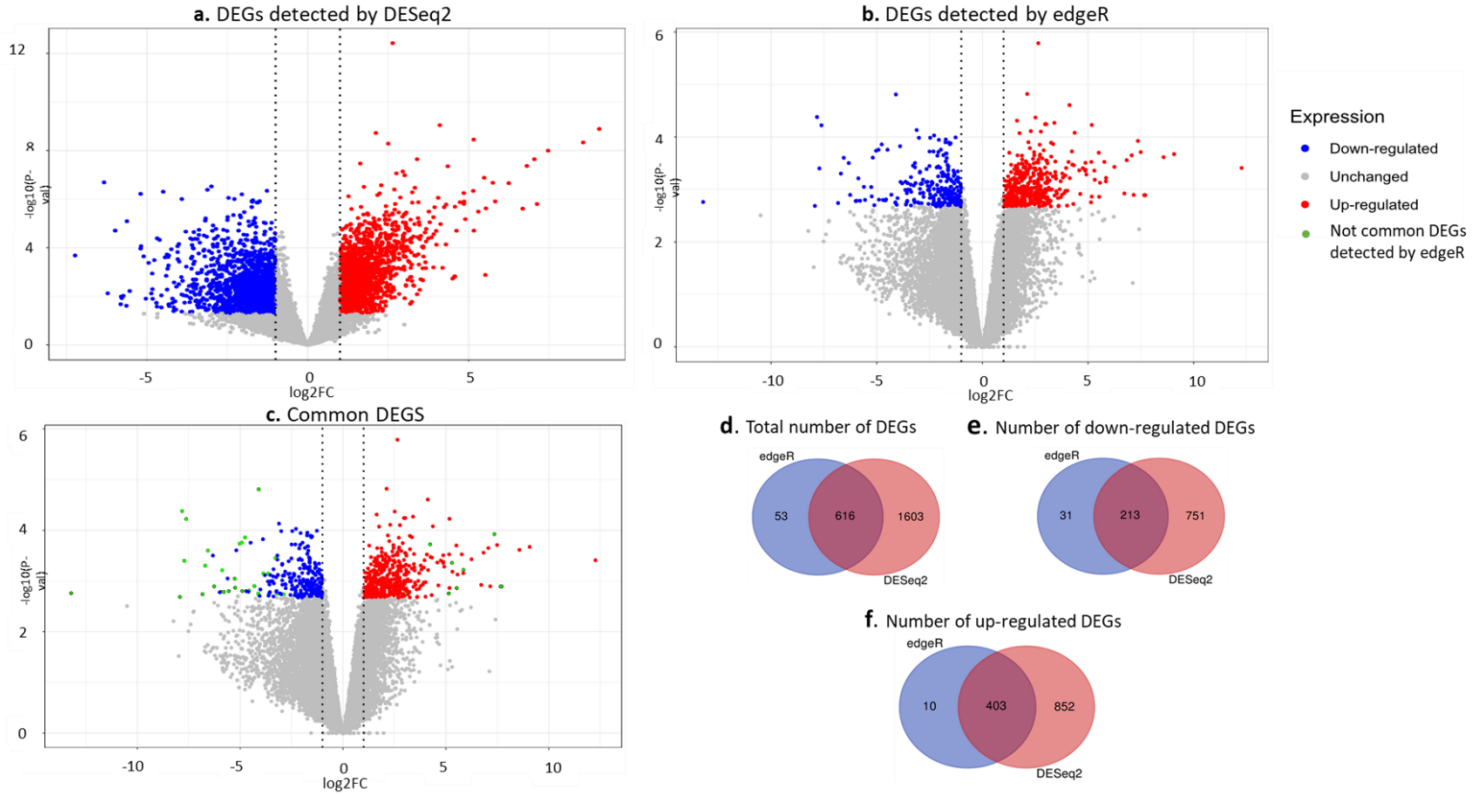


Figure 2. Volcano plots display differentially expressed genes (DEGs) distribution for DESeq2 (a), edgeR (b) and the consensous list between them (c). Red dots represent the up-regulated genes and blue dots the down-regulated. Grey dots indicate those DEGs not considered significant with p-value <= 0.05 and green dots show those DEGs that were not detected by both edgeR and DESeq2 methods. Venn diagrams representing the total number of DEGs detected by both edgeR and DESeq2 (d) and specific down- and up-regulated genes (e,f).

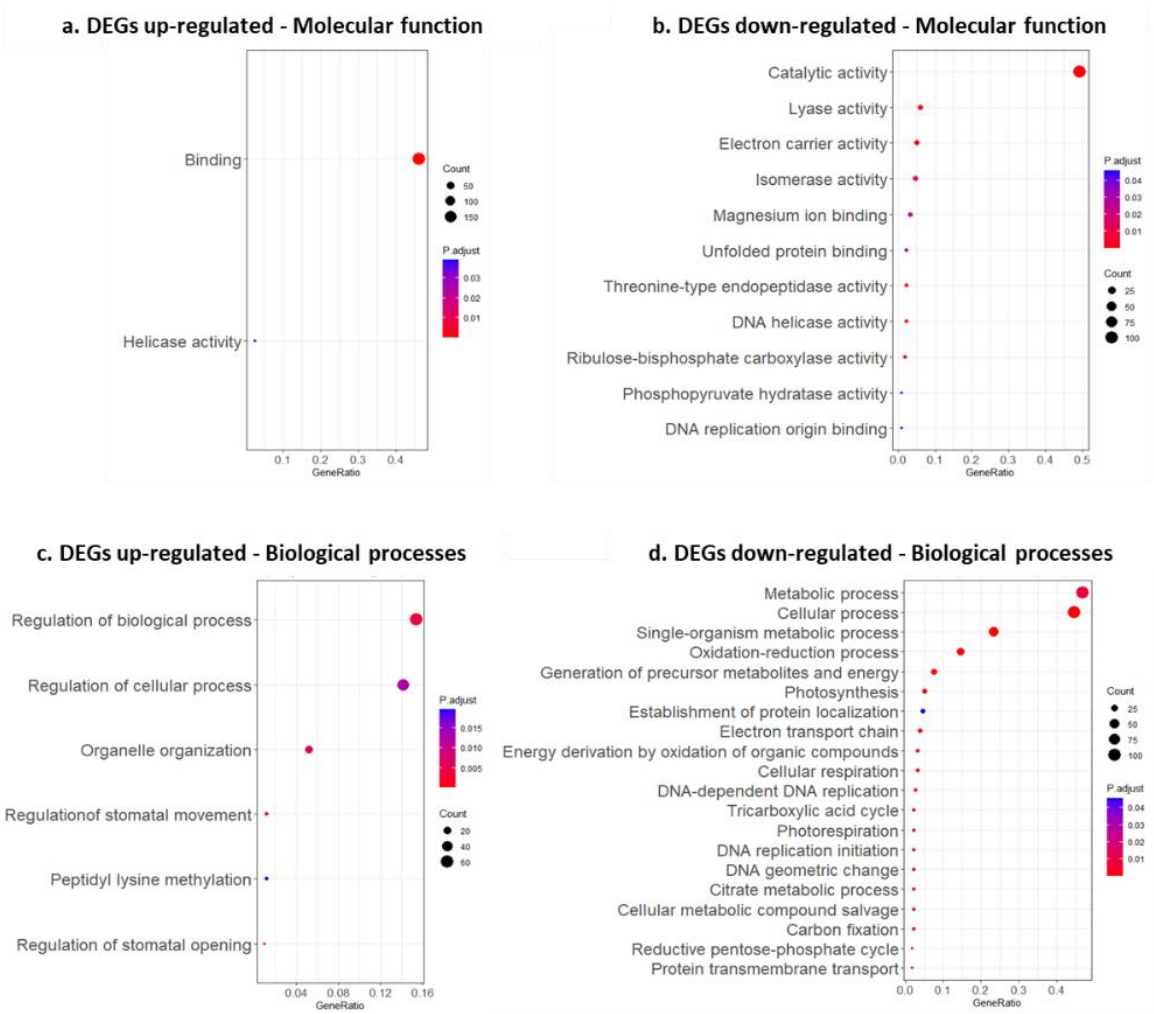


Figure 3. GO enrichment profile for up-(a,c) and down-represented genes (b,d). Enrichment in molecular functions (a, b), as well as, in biologicall processes (c, d) are indicated

2.4. Analysis of differentially expressed genes in the candidate regions for WMV resistance

2.4.1. Transcription changes on chromosome 11

The expression analysis was firstly focused on the region of the TGR-1551 genome associated to WMV resistance in chromosome 11, between positions 27,360,229 bp and 27,500,218bp (Pérez-de-Castro *et al.*, 2019). This QTL interval had been determined by using phenotypic data obtained in three different environments (LOD peaks values: 8.7, 11.8 and 10.8) and further fine mapped with descendance

tests (Pérez-de-Castro *et al.*, 2019). In this 140kb interval, there were 12 predicted genes, some of which had annotations related to plant defense responses. Three of these genes were found to be over-expressed in the resistant genotype and none of them was down-regulated (**Table 2**).

The highest induced expression was detected for a basic 7S globulin-like protein (MELO3C021406) with a log2FC (log2 fold change) of 3.95. A gene coding a dual specificity phosphatase 1 (MELO3C021395) was also up-regulated in the resistant RIL-10-3 with a log2FC of 1.82. The third up-regulated gene, with a log2FC of 1.02, was a mediator of RNA polymerase II transcription subunit (MELO3C021395).

Additionally, a *Vacuolar protein sorting 4* (*CmVps4,* MELO3C021413) has recently been proposed as a susceptibility factor related to the resistance against WMV derived from TGR-1551 (Agaoua *et al.*, 2022). This candidate gene is located out of the chromosome 11 candidate region proposed by Pérez-de-Castro *et al*. (Pérez-de-Castro *et al.*, 2019) and was not detected as differentially expressed in the RNA-seq.

Table 2. Predicted genes located within the candidate interval of the major QTL in chromosome 11. Log2(FoldChange) of the differentially expressed genes between the resistant and susceptible genotype for the interaction term genotype x treatment is provided.

Gene name	Position in chromosome 11 (start … end bp)	Description	log2(Fold Change)
MELO3C021407	27.358.921...27.362.202	Stem-specific protein TSJT1	
MELO3C021406	27.367.916…27.369.425	basic 7S globulin-like	3.95
MELO3C031841	27.368.901…27369.269	Unknown protein	
MELO3C021405	27.371.643…27.374.270	dual specificity protein phosphatase 1	1.82
MELO3C021404	27.393.549…27.396.064	Heavy metal-associated isoprenylated plant protein 21	
MELO3C021403	27.401.421…27.405.646	TVP38/TMEM64 family membrane protein slr0305-like	
MELO3C021400	27.428.704…27432.334	DUF21 domain-containing protein	
MELO3C021398	27.443.667…27.449.111	serine incorporator 3	
MELO3C035181	27.444.501…27.444.779	Unknown protein	
MELO3C021397	27.451.007…27.455.813	Ribosomal protein L34e superfamily protein	
MELO3C021395	27.460.592…27.473.546	Mediator of RNA polymerase II transcription subunit 33A	1.02
MELO3C021394	27.494.941…27.501.078	Mitogen-activated protein kinase	

2.4.2 Transcription changes on chromosome 5.

A minor QTL with modifier effects was located on chromosome 5 (chr5: 24,607,286-27,617,536 bp) (LOD peak value: 3.3) (Pérez-de-Castro *et al.*, 2019). As the candidate interval of this QTL was bigger than the one of the major QTL on chromosome 11, a larger list of DEGs was obtained. There were 11 and 5 significantly up- and down-regulated DEGs in the resistant RIL-10-3, respectively (**Table 3**) (**Suppplementary Table 1**). Among the up-regulated DEGs there were 7 of them whose annotations had been related to plant defense functions: an importin subunit alpha (MELO3C004204), a 5-3 exoribonuclease (MELO3C004356), a prenylyltransferase superfamily protein (MELO3C004366), a calcium uptake protein (MELO3C004433), a serine-rich protein-like protein (MELO3C004434), a transmembrane protein (MELO3C004435) and a ubiquitin family protein (MELO3C004438). On the other hand, the annotation of all the down-regulated genes in this region had been associated to responses to biotic stresses: a calreticulin protein (MELO3C004194), a GTP-binding protein SAR1A (MELO3C004196), a terpene cyclase/mutase family member (MELO3C004329), a thioredoxin-like protein (MELO3C004371) and a DNA helicase (MELO3C004448).

2.5. Expression of known genes related to plant defense responses.

The gene responsible of WMV resistance in *Arabidopsis thaliana* has been cloned and it encodes a nucleus-encoded chloroplast phosphoglycerate kinase (Ouibrahim *et al.*, 2014). Its ortholog gene in melon (MELO3C019634) was not deregulated and neither were genes with the same annotated function (**Additional Table 1**). Same happened with the genes related to WMV resistance in cucumber. In this crop, a QTL linked to the recessive gene wmv^{02245} was mapped on chromosome 6 (Tian *et al.*, 2016). The 134.7 kb physical distance of this region included 21 candidate genes, 16 of which were annotated. Five of those candidate genes were related to plant defense functions and included 2 zinc finger structures, 2 nucleic acid and protein binding sites and a pathogenesis-related transcriptional factor. The ortholog genes in melon were mainly located on chromosome 5 but they were not deregulated.

Table 3. Predicted genes located within the candidate interval of the major QTL in chromosome 5 that have been detected as differentially expressed. Log2(FoldChange) of the differentially expressed genes between the resistant and susceptible genotype for the interaction term genotype x treatment is provided

Gene name	Chr	Position (start … end bp)	Description	log2(Fold Change)
MELO3C004194	5	24,886,205…24,890,478	calreticulin	-1.64
MELO3C004196	5	24,898,167…24,901,559	GTP-binding protein SAR1A	-1.52
MELO3C004200	5	24,947,277…24,952,407	E3 SUMO-protein ligase NSE2	1.48
MELO3C004204	5	24,983,611…24,989,254	Importin subunit alpha	2.15
MELO3C004219	5	25,131,665…25,134,287	Dormancy/auxin associated protein	3.16
MELO3C004305	5	26,042,063…26,048,099	Pre-mRNA-splicing factor SLU7	2.97
MELO3C004329	5	26,298,777…26,308,596	Terpene cyclase/mutase family member	-2.59
MELO3C004356	5	26,610,676…26,621,447	"5-3 exoribonuclease"	1.14
MELO3C004366	5	26,689,354…26,697,690	Prenylyltransferase superfamily protein	2.15
MELO3C004371	5	26,731,110…26,735,263	Thioredoxin-like protein 1	-1.24
MELO3C004421	5	27,112,362…27,117,173	L-allo-threonine aldolase	4.36
MELO3C004433	5	27,203,527…27,207,131	calcium uptake protein 1, mitochondrial-like isoform X1	5.18
MELO3C004434	5	27,208,137…27,211,375	Serine-rich protein-like protein	4.12
MELO3C004435	5	27,217,111…27,221,085	Transmembrane protein	1.88
MELO3C004438	5	27,252,281…27,262,164	Ubiquitin family protein	2.30
MELO3C004448	5	27,326,640…27,332,555	DNA helicase	-4.49

To further identify candidate genes related to WMV resistance, the expression profile of genes associated with plant defense responses against viruses were studied. We looked for R-genes previously characterized in melon (Islam *et al.*, 2020), families of transcription factors (TFs) involved in stress responses, pathogen-resistant proteins, genes involved in gene silencing and hormonal signaling and susceptibility factors (**Figure 4**).

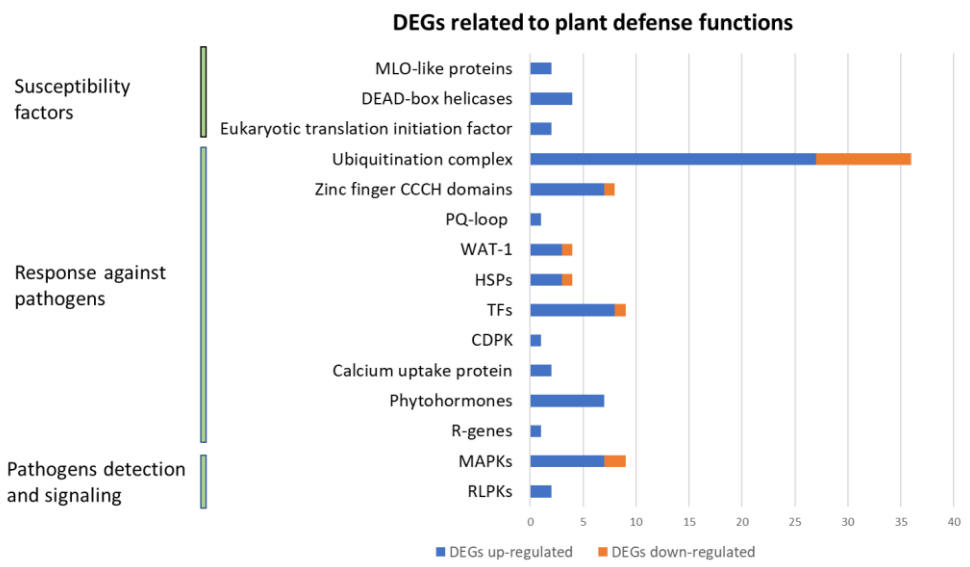


Figure 4. Number of differentially expressed genes (DEGs) up- and down-regulated whose annotation has previously been related to different plant defense functions

Among the 70 characterized R-genes in *C. melo* (Islam *et al.*, 2020), none of them were deregulated. Neither were an additional set of selected genes conferring resistance to pathogens (Sáez *et al.*, 2022a). In other resistance studies carried out in cucurbits, these resistance genes were mostly deregulated at latter infection stages (6 and 12 dpi) (Lou *et al.*, 2020; Sáez *et al.*, 2022a). On the other hand, protein kinases are known to mediate the signaling mechanisms required for the defense response, including the activation of TFs and systemic responses. Among the up-regulated DEGs there was one located in chromosome 5 coding a disease resistance protein (MELO3C008572) (**Additional Table 1**), seven genes coding mitogen-activated (MAPKs) or serine/threonine protein kinases (MELO3C021470, MELO3C012233, MELO3C013739, MELO3C010334, MELO3C019687, MELO3C013322 and MELO3C026640), as well as two receptor protein-kinases (RPKs) (MELO3C002351 and MELO3C007457) and a calcium-dependent protein kinase (CDPK) (MELO3C017756) (**Figure 4**). Many other kinases were also

overexpressed (**Additional Table 1**). Among the down-regulated DEGs, there were two genes coding a mitogen-activated or serine/threonine-protein kinase (MELO3C026848 and MELO3C003047) and a dual specificity phosphatase (MELO3C024481).

There were other DEGs whose functional annotation had also been described as related to plant defense responses. Among these genes there were heat-shock proteins (HSP) (MELO3C007297, MELO3C016712, MELO3C021100 and MELO3C008865), which are molecular chaperones that protect plants from damage to diverse stresses; WAT1-related proteins (MELO3C010177, MELO3C010471, MELO3C009934 and MELO3C012015) that have been associated to resistance against the *cucumovirus* cucumber mosaic virus (CMV) in pepper (Zhu *et al.*, 2018b); or protein domains that form part of some atypical R proteins, such as PQ-loop repeat proteins (MELO3C020942) or zinc finger CCCH domains (Deng *et al.*, 2012; Liu & Wang, 2016; Pi *et al.*, 2018; Tyagi *et al.*, 2014) (**Figure 4; Additional Table 1)**.

2.5.1. Transcription factors

Among the 58 transcription factors (TFs) families described in plants, six major TFs families have been reported as involved in stress responses (Ng *et al.*, 2018). We looked for DEGs coding TFs implied in plant-defense functions and two ERFs (ethylene responsive transcription factors) (MELO3C007572 and MELO3C021306), two bHLH (basic helix-loop-helix) (MELO3C023299 and MELO3C005178), two bZIP (basic leucine zipper) (MELO3C012961 and MELO3C015377), one MYB (myeloblastosis related) (MELO3C024440) and one NAC (no apical meristem (NAM)) (MELO3C012391) were up-regulated, while only one MYB was down-regulated (MELO3C012039). Thus, a strong de-regulation of the TFs expression profile was confirmed in the resistant genotype even at early infection stages.

2.5.2. Hormones

Phytohormones, including ethylene (ET), salicylic acid (SA), jasmonate (JA), abscisic acid (ABA), brassinosteroids (BR), cytokinin (CK) or auxins (AUX) play essential roles by activating the plant defense response against viruses. We looked for DEGs coding proteins related to phytohormone response or synthesis. In addition to the previously named TFs affected by JA or ET accumulation (see previous section), two genes coding stem-specific protein TSJT1 (MELO3C007297 and MELO3C016712) were up-regulated. These proteins participate in the systemic acquired resistance (SAR) through the SA mediated signaling pathway (Spagnolo *et al.*, 2014). There was

also an over-expressed gene coding a glycosyltransferase (MELO3C009339), which could be involved in the SA cycle by modulating N-hydroxy-pipecolic acid (NHP), a regulator for plant innate immunity and SAR (Mohnike *et al.*, 2021). Finally, four genes coding proteins related to AUX were also over-expressed (**Additional Table 1**) (**Figure 4).**

2.5.3. Ubiquitination and ubiquitin/proteasome system complex

The ubiquitin proteasome system (UPS) constitutes an important part of plant responses to viruses. It can target different viral components to prevent virus spread, inhibit viral replication or to mitigate disease symptoms (Dubiella & Serrano, 2021; Verchot, 2016). There were 27 over-expressed genes encoding proteins that are part of the UPS. Among the coded proteins there were 4 ubiquitin-conjugation enzymes, 11 ubiquitin-protein ligases (including RING-type) and 9 F-box proteins. RING-type ligases and F-Box proteins are UPS key factors, since they, respectively, define the substrates for ubiquitination and induce the hypersensitive resistance response (Van Den Burg *et al.*, 2008). There were 7 and 2 additional DEGs that were down-regulated and coded different proteasome subunits and F-box proteins, respectively (**Figure 4**) (**Additional Table 1**).

2.5.4. RNA silencing

RNA silencing constitutes an important defense method against viral infections. Even though potyvirus code a HC-Pro silencing suppressor that puts down the miRNA pathway (Chapman *et al.*, 2004), it has been proposed that silencing may play a key role in the resistance response of TGR-1551 to WMV (Gonzalez-Ibeas *et al.*, 2011). We searched for DEGs involved in this mechanism but there were not DEGs coding proteins directly implied in the silencing machinery, such as DICER-like genes, ARGONAUTE genes or genes involved in the RNA-induced silencing complexes.

2.5.5. Susceptibility factors

Cellular translation factors are recruited by plant viruses to both translate their viral RNAs and to control their replication and movement through the plant. Hence, mutations in these proteins can lead to broad spectrum resistances (Mazier *et al.*, 2011; Rodríguez-Hernández *et al.*, 2012). Additionally, other translation factors such as eIF4A-like helicases (a DEAD-box ATP- dependent RNA helicase) are also frequently used by viruses (Sanfaçon, 2015) and can act as effectors by blocking RNA virus replication (Wu & Nagy, 2019). Genes coding one eukaryotic translation initiation factor-like protein (MELO3C002515), one translation initiation factor 4E

(MELO3C026612) and four DEAD-box ATP-dependent RNA helicases (MELO3C023052, MELO3C009973, MELO3C017907, MELO3C006599) were over-expressed in the resistant RIL 10-3 compared to the susceptible cultivar BO (**Figure 4)** (**Additional Table 1**). There were not translation factors downregulated.

Moreover, *CmVps4* (MELO3C021413) and *CmVps41* (MELO3C004827) had been proposed as a susceptibility factors conferring resistance against WMV and CMV systemic infections in melon (Agaoua *et al.*, 2022; Giner *et al.*, 2017; Pascual *et al.*, 2019) . Additionally, a VPS4-like gene has also been proposed as a candidate gene conferring resistance to zucchini yellow mosaic virus (ZYMV) in cucumber (Ramírez-Madera & Havey, 2017). Nevertheless, those genes were not detected as DEGs in the RNA-seq study. Only two genes annotated as vacuolar protein sorting-associated proteins (MELO3C005953 and MELO3C032233) were up-regulated in the RIL-10-3 (**Additional Table 1**).

2.6. qRT-PCR validation of DEGs.

In total, 6 significant differentially expressed genes were selected for qRT-PCR validation (**Figure 5**). The selected genes were located on chromosomes 11 (MELO3C021395, MELO3C021406 and MELO3C021413) and 5 (MELO3C004433, MELO3C004448, MELO3C004204).

The normalized relative accumulation of their transcripts measured by RT-qPCR was compared to the number of lectures detected by RNA-seq, showing similar expression patterns relative to the genotype x treatment term. The ANOVA tests showed that there were significative differences for the genotype x treatment interaction term for the 6 studied genes (p.value < 0.05). This is consistent with the results obtained for the RNA-seq analysisof all the genes (**Table 2** and **Table 3**) except for MELO3C021413, which was not detected as differentially expressed in the transcriptomic assay.

However, when the expression patterns of both RNA-seq and RT-qPCR data were compared a similar expression trend was observed. The differential expression analysis conducted with the RNA-seq data considers the expression profiles of all the genes in the genome to determine if each one of them is differentially expressed. This can lead to underestimate the differences, which explains the significance differences between both methods when a similar expression trend is observed. The RT-qPCR results confirmed the high reproducibility of the obtained transcriptomic data.

Figure 5. Comparation of the expression profiles of 6 candidate genes for WMV resistance obtained by both RNA-seq and RT-qPCR. Left panels of each gene represent the number of lectures detected by RNA-seq, while right pannels indicate their relative expression (dCt)determined by RT-qPCR. The central line within the box represents the median expression value. The box encompasses the interquartile range (IQR). The whiskers extending from the box indicate the minimum and maximum values within 1.5 times the IQR. Data points beyond the whiskers are represented as individual points and are considered outliers.

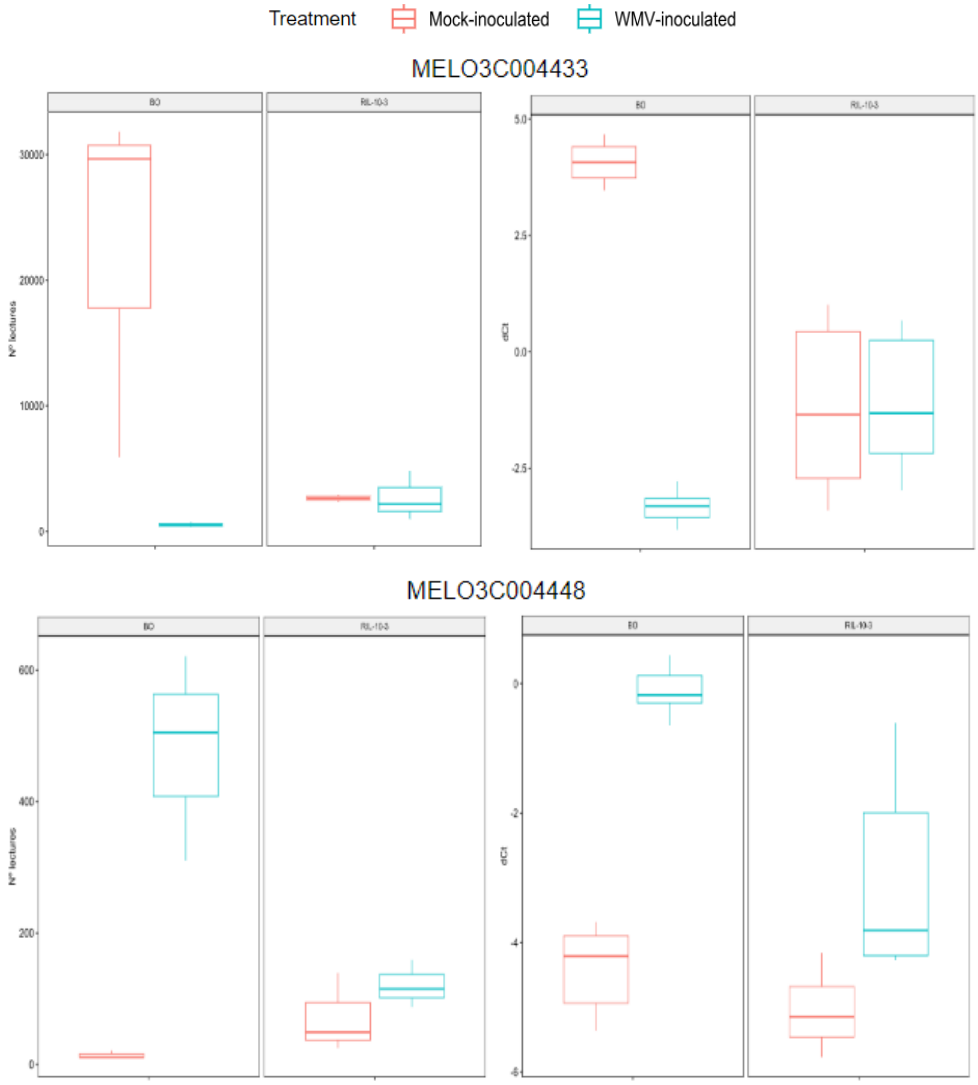


Figure 5. Continuation

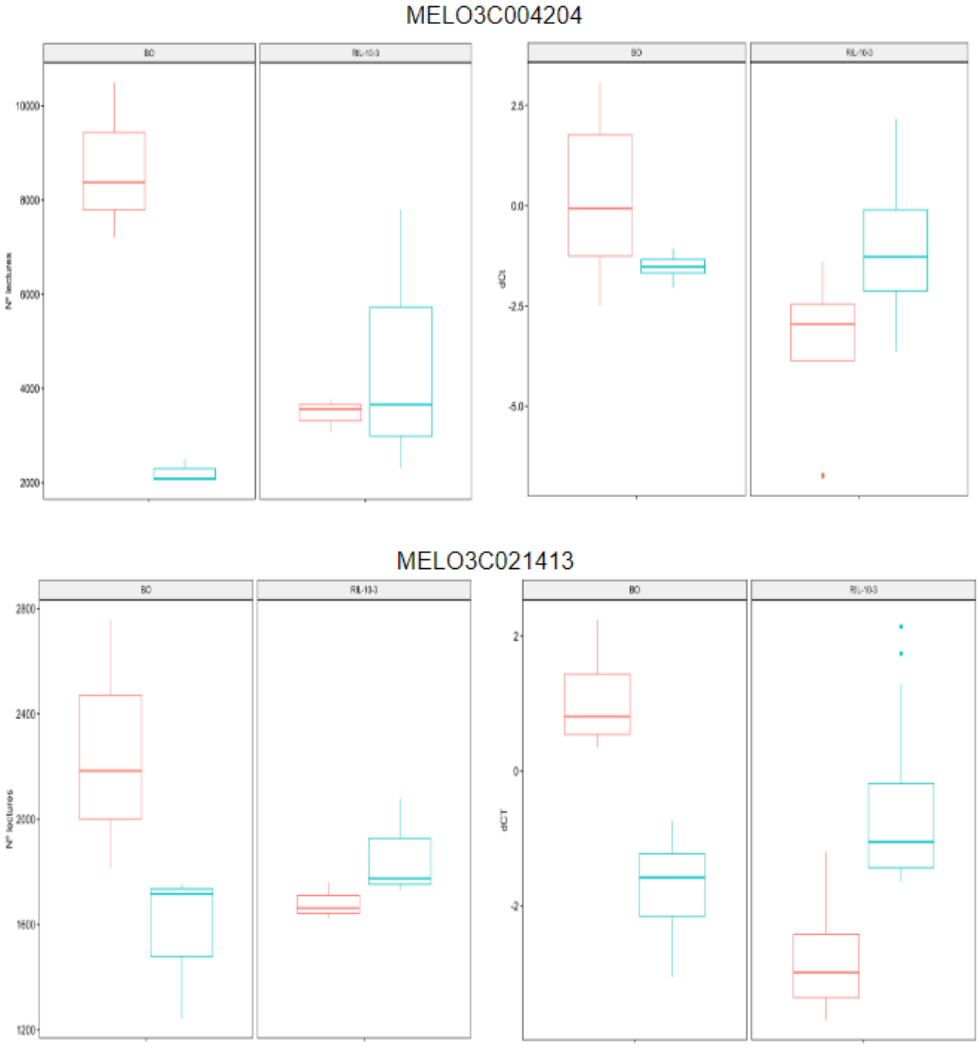
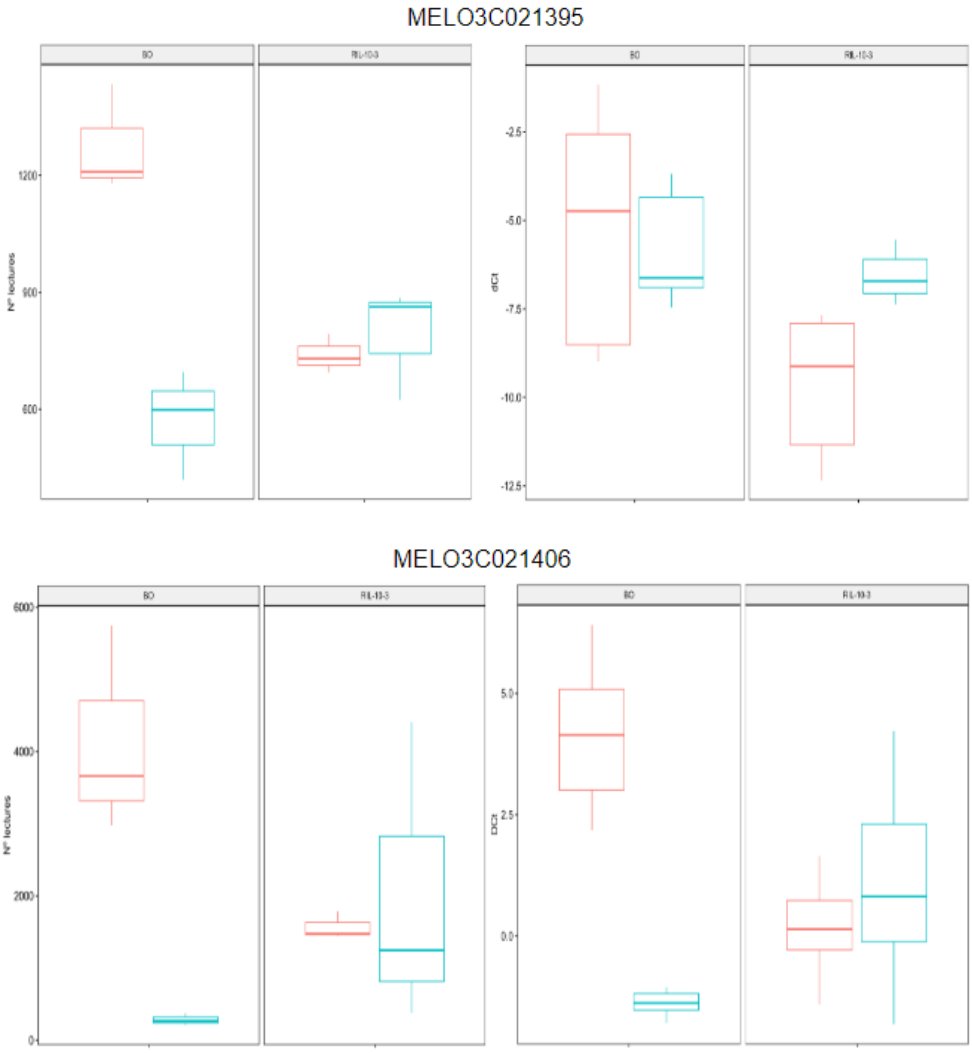


Figure 5. Continuation



2.7. SNPs linked to DEGs associated with WMV response

Out of the 198,881 variants detected between the RIL-10-3 and BO samples, multiallelic SNPs were discarded, so only 192,561 were considered. There were 303,694 (75.26%), 23,171 (5.74%), 47,764 (11.84%) and 28,874 (7.16%) variants with a modifier, moderate, low and high predicted impact, respectively (**Figure 6**). Of the 28,874 variants with a predicted high impact effect, 9,708 (33.63%) were associated to gene coding sequences. Moreover, SNPs were detected in 401 of the 403 (99.5%) DEGs that were up-regulated, and 334 of those variants were

associated with a predicted high impact effect (i.e., appearance of codon stops, open reading frame shifts, amino acid changes not favored by evolution…). Regarding to the 213 DEGs that were down-regulated, SNPs with a high impact were detected in 152 of them (71,36%).

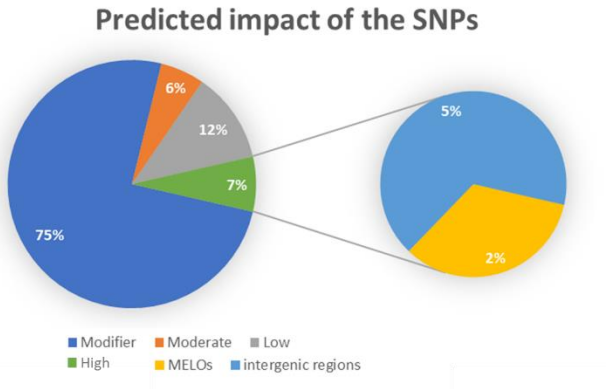


Figure 6. Representation of the percentage of detected SNPs with different predicted impacts on the protein function.

SNPs variants with a high predicted impact were observed in the three DEGs detected in the candidate region of chromosome 11 (MELO3C021405, MELO3C021406 and MELO3C021395) (**Table 3**). Among others, those effects were due to the gain of a premature start codon gain, the appearance of a missense variant causing an aminoacidic change and modifications of the splicing regions. The gen MELO3C021395 (MED33A) was the one in which more high-impact variants were detected (**Table 3**). Additionally, SNPs were detected in the coding regions of all the genes located within the candidate interval. As for the *vacuolar protein sorting 4* (MELO3C021413), proposed as a susceptibility factor, 6 high impact variants were observed. Nevertheless, it had been proposed that a single non-synonymous substitution in *CmVps4*^{P30R} conferred the resistance to WMV (Agaoua *et al.*, 2022). This mutation is caused by a [T/C] SNP in the genomic position Chr11:27,319,260 bp (v.4.0) but both BO and the RIL-10-3 have a cytosine in this position. These GBS results were also confirmed by sequencing by Sanger this genomic region of BO, TGR-1551 and RIL-10-3, obtaining the same result. The presence of a cytosine in this genomic position is translated into a proline (P), instead of into an arginine (R). Thereby, the previously described change in TGR-1551 *CmVps4*^{P30R} was not present in our resistant accession.

Regarding the DEGs identified within the QTL candidate region in chromosome 5, SNPs with a high predicted impact were detected in all of them except for the genes MELO3C004329, MELO3C004356 and MELO3C004434.

Moreover, at least one SNP with a predicted high effect hit all the previously mentioned DEGs except for the down-regulated MYB transcription factor (MELO3C012039), that was affected by variants with low, moderate and modifier effects. These changes in the genomic sequences could affect the expression of those genes and the activity of the coded proteins.

Table 3. Summary of the SNPs observed within the sequences of the genes located in the candidate region of chromosome 11 and among the DEGs detected within the QTL in chromosome 5. The predicted impact and the effect of the SNPs is indicated.

			Variants impact				Variants effect						
Gene name	Chr	log2(Fold Change)	High	Low	Moderate	Modifier	Premature start codon gain	Premature stop codon gain	Frameshit variant	Missense variant	Splice acceptor variant	Splice donor variant	Splice region variant
MELO3C004194	5	-1.64	3	3	2	12	0	0	0	1	0	3	6
MELO3C004196	5	-1.51	2	8	1	20	0	0	1	1	0	1	7
MELO3C004200	5	1.47	2	5	5	8	0	0	0	5	0	2	2
MELO3C004204	5	2.15	8	15	2	21	1	1	1	2	2	5	15
MELO3C004219	5	3.16	3	2	1	22	1	1	0	1	1	1	3
MELO3C004305	5	2.96	5	3	0	27	0	0	0	0	1	4	6
MELO3C004329	5	-2.59	0	0	2	33	0	0	0	2	0	0	0
MELO3C004356	5	1.13	0	3	2	30	1	0	0	2	0	0	1
MELO3C004366	5	2.15	3	3	0	29	0	0	0	0	1	2	4
MELO3C004371	5	-1.24	1	0	0	37	0	0	1	1	1	0	1
MELO3C004421	5	4.36	4	6	4	32	0	0	0	4	1	3	8
MELO3C004433	5	5.18	7	9	7	102	1	1	3	9	2	2	6
MELO3C004434	5	4.12	0	0	1	101	0	0	0	1	0	0	0
MELO3C004435	5	1.88	12	8	7	17	1	0	2	9	4	7	12
MELO3C004438	5	2.3	10	16	2	11	0	1	1	3	5	5	21
MELO3C004448	5	-4.49	1	1	0	13	0	0	0	0	0	1	1
MELO3C021407	11		2	7	1	10	0	0	1	2	0	1	6
MELO3C021405	11	1.82	1	2	1	27	1	0	0	1	0	1	1
MELO3C021406	11	3.95	1	2	8	15	0	0	1	7	0	0	0
MELO3C031841	11		0	0	3	23	0	0	0	3	0	0	0
MELO3C021404	11		3	9	12	10	0	0	2	7	1	0	2
MELO3C021403	11		0	2	1	5	0	0	0	1	0	0	1
MELO3C021400	11		2	5	1	19	0	0	0	1	0	2	5
MELO3C035181	11		0	0	1	15	0	0	0	1	0	0	0
MELO3C021398	11		1	5	2	16	0	0	0	2	0	1	3
MELO3C021397	11		0	0	2	37	0	0	0	2	0	0	0
MELO3C021395	11	1.02	9	12	10	58	0	0	2	11	3	4	8
MELO3C021394	11		8	15	0	26	0	0	0	0	2	6	18

2.8. Weighted gene co-expression network analysis.

Weighted gene co-expression network analysis (WGCNA) produced 19 gene clusters (GCs) (**Additional Figure 1**). GC6 cluster was the only one showing a genotype-specific pattern of gene co-expression. Moreover, there were seven clusters (GCs 1, 9, 10, 13, 14, 17 and 19) that showed statistically significant differences regarding the interaction term between genotype and treatment. Most up-regulated genes in the RIL-10-3 within these GCs were enriched in KEGG pathways associated to plant hormone signaling transduction, MAPK signaling pathway, spliceosome, ubiquitination, and basal transcription factors, whereas the genes that had an expression profile negatively correlated with the clusters were mainly classified in ontologies related with replication, photosynthesis, carbon metabolism, including the TCA cycle, and ribosome (**Additional Figure 2**).

3. Discussion

WMV is one of the most limiting factors for melon production worldwide, as it affects all the main producing areas. Moreover, this potyvirus is constantly evolving, and new and more virulent strains continue to appear (Bertin *et al.*, 2020; Desbiez *et al.*, 2009, 2020). The African accession TGR-1551 is the most promising resistance source against WMV. In works developed by our research group, a major QTL related to the recessive resistance was mapped to a 140 kb region, containing 12 predicted genes, in chromosome 11. Additionally, a minor QTL with modifying effects was also mapped to a wider region in chromosome 5 (Pérez-de-Castro *et al.*, 2019). Previous microarray studies revealed a huge transcriptomic remodeling related to this resistance (Gonzalez-Ibeas *et al.*, 2012) and a RNAome assay highlighted the possibility that silencing mechanisms could also be implied in the immune response (Gonzalez-Ibeas *et al.*, 2011). In this study, a resistant and a susceptible genotype sharing approximately a 50% of their genome were mock- and virus-inoculated, and we took advantage of the assets offered by RNA-seq compared to microarrays, to take a closer look at the transcriptional changes after WMV infection, trying to correlate the early changes in gene expression to the resistance response.

When we compared the genotype x treatment interaction term, 616 common DEGs were obtained with two different algorithms, edgeR and DESeq2. Out of those 616 genes, 403 and 213 were up- and down-regulated, respectively. This is consistent with the huge transcriptomic remodeling previously observed after inoculation of resistant genotypes with WMV (Gonzalez-Ibeas *et al.*, 2012), ZYMV (Amoroso *et al.*, 2021) and tomato leaf curl New Delhi virus (ToLCNDV) (Sáez *et al.*, 2022a),

respectively. Moreover, 3 and 16 DEGs were identified within the major and minor QTL regions in chromosomes 11 and 5, respectively.

In the chromosome 11 region, the 3 DEGs were all up-regulated in the resistant RIL-10-3. Among them, the gene with the highest fold change difference presented a frameshift mutation in the RIL-10-3, and coded a basic 7S globulin-like protein (Bg7S; MELO3C021406). Even though Bg7S were initially thought to only be seed storage proteins, they have been proven to be multifunctional (Hirano, 2021). In tomato, Bg7S inhibits xyloglucan-specific endo-β-1,4-glucanase (XEG), a cell wall-degrading glucosyl hydrolase derived from *Aspergillus aculeatus* (Qin *et al.*, 2003). In soybean, Bg7S is expressed in response to biotic and abiotic stressors and it has been shown to have protein kinase activity (Komatsu *et al.*, 1994). However, despite the multiple effects described for this protein, to our knowledge, Bg7S have not previously been related to resistance responses against viruses.

Another DEG located within the candidate region in chromosome 11 coded a dual specificity phosphatase 1 (MELO3C021395), and 9 variants with a predicted high impact were found within its sequence. A gene with the same predicted function was also found in the WMV resistance candidate region in cucumber (Tian *et al.*, 2016). Dual specificity phosphatases are a sub-class of MAPK phosphatases (MKPs) whose main function is to ensure an appropriate balance stress signaling and suppression of autoimmune-like responses by negatively modulating the MAPK kinetics (Ayatollahi *et al.*, 2022; Bartels *et al.*, 2009; Gupta *et al.*, 1998; Jiang *et al.*, 2018; Shubchynskyy *et al.*, 2017). They are involved in controlling plant growth and development as well as modulating stress adaptation (Jiang *et al.*, 2018). Some dual-phosphatases also modulate phytohormone signal transduction pathways, especially those related to auxins, SA and ABA (Bartels *et al.*, 2009; Monroe-Augustus *et al.*, 2003; Quettier *et al.*, 2006;). Nevertheless, these proteins have always been associated with higher resistance levels when their expression is repressed or by avoiding autoimmune damages. Contrary to what was expected, in this assay this gene was over expressed in the resistant genotype, and no necrotic damages associated with the immune response have been reported in the susceptible cultivars.

The third gene that was up-regulated in the candidate region of chromosome 11 coded a mediator of RNA polymerase II transcription subunit 33A (MED33A; MELO3C021395). A premature start codon gain was detected within its coding sequence. Mediator is a large multi-subunit complex that integrates input signals from different pathways and connects them to the RNA polymerase II (RNAPII). Mediator complex plays a key role in fine-tune pathway- and gene-specific

transcriptional reprogramming by acting as a hub between TFs and RNAPII (Malik & Roeder, 2010). MED33A (REF4-related 1; RFR1) and MED33B (reduced epidermal fluorescence 4; REF4) subunits are implied the regulation of the phenylpropanoid pathway (PPP), acting as repressors (Ruegger & Chapple, 2001; Stout *et al.*, 2008). Knock-out mutants of the MED33A and MED33B subunits showed an increased expression of genes such as phenylalanine-ammonia lyase 1 (PAL1), PAL2, cinnamate 4-hydroxylase (C4H) and 4CL1, that are implied in the early phenylpropanoid biosynthetic pathway (Bonawitz *et al.*, 2012). PPP-derived metabolites play diverse roles in plant defense and are often positively correlated with resistance. In fact, downstream of the core PPP, accumulation of PPP-derived phytoalexins are common resistance mechanisms (Chezem *et al.*, 2017; Desmedt *et al.*, 2020; Dixon *et al.*, 2002; Obermeier *et al.*, 2013; Ranjan *et al.*, 2019; Veronico *et al.*, 2018) . Nevertheless, the perturbation of the PPP through the application of the C4H inhibitor piperonylic acid (PA) in tomato triggered systemic, broad-spectrum resistance by systemically inducing immune signaling and pathogenesis-related genes and locally activating the production of reactive oxygen species (ROS) (Desmedt *et al.*, 2021). Thus, the over-expression of MED33A in the resistant RIL-10-3 and the consequent repression of the PP synthesis could lead to an early strong defense response to WMV.

Moreover, several mediator subunits are directly related to plant defense functions by both relaying signals from upstream regulators and by transmitting phytohormone signals (An & Mou, 2013; Zhai & Li, 2019). Some mediator subunits such as MED18 and MED25 have been directly related to virus defense in *A. thaliana*. Both subunits are implied in the JA signaling pathway but their silencing affects virus infection differently. Whereas MED18 is considered a susceptibility factor, MED25 is required for defense against virus infection. The up-regulation of MED33 in the resistant genotype could indicate that this subunit would also be implied in an active defense response against WMV. Recently, a *Vacuolar sorting 4* (*CmVps4*) (MELO3C021413) has been proposed as a susceptibility factor to WMV in melon (Agaoua *et al.*, 2022). This gene was located out of the chromosome 11 candidate region proposed by Pérez-de-Castro *et al*. (Pérez-de-Castro *et al.*, 2019). It was not deregulated in the RNA-seq assay, and the RT-qPCR data showed that it was slightly up-regulated in the resistant RIL-10-3 which would be contrary to the expression patterns showed by other susceptibility factors, where this kind of genes are over-expressed in the susceptible genotypes under infection conditions (Siskos *et al.*, 2023; Zhang *et al.*, 2023). Moreover, we observed that both TGR-1551 and RIL-10-3 genotypes did not have the mutation $CmVps4^{P30S}$ that is supposed to confer the resistance. Instead TGR-1551, RIL-10-3 and BO carried the $Vps4^{Wt}$ allele,

which has also been observed in the resistant accession PI 414723 but that is not related to the resistance derived from TGR-1551. These discrepancies between the TGR-1551 genotypes could be explained by the fact that this wild relative was found in open-pollinated populations, were the level of heterogenicity is higher and different processes of self-pollination have resulted in these differences. Either way, it does not seem likely that *CmVps4* by itself could be responsible of the WMV resistance derived from TGR-1551. Other major resistance mechanisms could be implied in the defense response.

Within the chromosome 5 candidate region there were also several DEGs whose annotated functions had previously been associated to resistance responses against pathogens. Ubiquitin family proteins (MELO3C004438) can target different viral components to prevent virus spread, inhibit viral replication or to mitigate disease symptoms (Czosnek *et al.*, 2013; Siskos *et al.*, 2023). Transmembrane proteins (MELO3C004435) and importins (MELO3C004204) can act as susceptibility factors and have also been related to silencing responses (Chen *et al.*, 2021; Parween & Sahu, 2022; Peng *et al.*, 2022; Zhai *et al.*, 2021). Exoribonucleases (MELO3C004356) can negatively regulate the accumulation of viruses (Pan, 2019; Pouclet *et al.*, 2023). Calreticulins (MELO3C004194) are a kind of chaperones that binds to calcium and have been associated to the defense response against biotrophic pathogens (Joshi *et al.*, 2019; Pröbsting *et al.*, 2020). They are essential to the correct maturation of some surface glycosylated receptors. The gen coding a terpeno cyclase/mutase family member (MELO3C004329) was down-regulated in our resistant genotype, but these kind of genes have been found to be over expressed in the resistant accession WM7 when it was inoculated with ToLCNDV (Sáez *et al.*, 2022a). As it happened with MED33A, these proteins are implied in the PPP (Bosamia *et al.*, 2020). A gene coding a thioredoxin-like protein (Trxs) (MELO3C004371) was also down-regulated in the RIL-10-3. Trxs can contribute to plant defense by expressing defense responsive pathogenesis-related (PR) genes (Kumari *et al.*, 2021) but have also been described as negative regulators of ROS production (Leonetti & Pantaleo, 2021; Liu *et al.*, 2021). Finally, calcium uptake proteins (MELO3C004433), GTP-binding proteins (MELO3C004196) and serine-rich proteins (MELO3C004434) have been related to the transduction of signals after the recognition of pathogen associated molecular patterns (PAMPs) (Blume *et al.*, 2000; Lecourieux *et al.*, 2006; Upasani *et al.*, 2017; Shi *et al.*, 2021; Xu *et al.*, 2021). Moreover, calcium signals also play an important role in the second layer of defense called effector-triggered immunity (ETI) (Grant *et al.*, 2000). It has been observed that the calcium signal can be downstream translated into outputs such as gene expression or stomatal closure (Thor, 2019). In this sense, it is worth saying that the

regulation of stomatal movement and opening were two of the enriched biological processes among the DEGs that were up-regulated. The expression pattern differences between the resistant and susceptible genotypes might be due to the accumulation of SNPs with a predicted high impact in the coding regions of all the cited DEGs. Moreover, the minor QTL on chromosome 5 is located within a resistance cluster (Garcia-Mas *et al.*, 2012; Pérez-de-Castro *et al.*, 2019). This could explain the huge transcriptomic remodeling observed in this region in the resistant genotype after WMV-infection. As several genes could work in a synergistic manner to improve the resistance offered by the major gene on chromosome 11.

In addition to the changes observed within the two candidate regions, a huge transcriptomic remodeling was also observed across the whole genome, which is consistent with previous microarray data (Gonzalez-Ibeas *et al.*, 2012). Even though the gene ontologies of the detected DEGs were not enriched in plant defense related functions, numerous genes previously classified as susceptibility factors, or related to the pathogen's detection and transduction of signals and the response against viruses were differentially expressed. This is consistent with the fact that several gene clusters detected with WGCNA were enriched in molecular functions such as `plant-pathogen interaction´, `plant hormone signal transduction´, `MAPK signaling pathway´, `basal transcription factors´ or `ubiquitin mediated proteolysis´. The up-regulation of these biological processes had also been observed in other cucurbits infected with viruses (Amoroso *et al.*, 2021; Li *et al.*, 2017; Sáez *et al.*, 2022a; Sun *et al.*, 2017;). Moreover, both the list of DEGs and some GCs that were down-regulated in the resistant genotype were enriched in gene ontologies related to the photosynthesis and the basal metabolism. The activation of defense responses is energetically expensive, which is why it is often done to the detriment of photosynthesis and the assimilatory metabolism, specially at early infection stages (Scharte *et al.*, 2005). In summary, these results showed that an active fight against WMV is taking place in the resistant RIL-10-3.

As it has been stated before, plant immune responses to pathogens lead to a complex net of recognition, transduction and translation of signals that finally turns into the activation of different resistance mechanisms. After the recognition of the pathogen, one of the first steps in the immune pathway is the activation of MAPK (Jones & Dangl, 2006). In this work, the weighted gene co-expression analysis revealed that two statistically significant gene clusters (GC13 and GC14) were enriched in up-regulated genes classified in the `MAPK signaling pathway´ KEGG pathway. Moreover, seven and two DEGs coding MAPK were up- and down-regulated, respectively, in the resistant RIL-10-3 when compared to BO.

Additionally, two receptor-like kinases (RLKs) were also up-regulated. The activation of RLKs and the other MAPK could be directly involved in the resistance response. RLKs are implied in sensing diverse stimuli, which can both be endogenous (hormones o peptide-ligands) or exogenous signals (PAMPs) acting as pattern recognition receptors (Jones & Dangl, 2006). Meanwhile, MAPKs are key downstream signaling modules of RLKs. MAPKs play a key role in plant immune signaling, as it can be reflected by the fact that many pathogens' effectors target host MAPKs pathways (Feng & Zhou, 2012; King *et al.*, 2014; Zhang *et al.*, 2007). MAPKs can phosphorylate different substrates, such as TFs that would further be involved in hormone synthesis or the activation of genes involved in the resistance response, for example R genes.

In line with this, eight DEGs coding TFs related to stress responses were up-regulated in the RIL-10-3. Moreover, in the WGCNA, the KEGG term `basal transcription factors´ was enriched in the up-regulated genes of three gene clusters (GC13, GC14 and GC19). TFs are involved in a wide range of functions such as plant growth and development or stress responses. They can switch on the antiviral basal immunity through the interaction with the viral coat protein (Donze *et al.*, 2014). TFs can also interact with resistance proteins that impair viral propagation or with MAPKs and phytohormones that transduce the stress signal (Gutterson & Reuber, 2004; Fischer & Dröge-Laser, 2004; Huang *et al.*, 2016; Liu *et al.*, 2004; Yu *et al.*, 2012; Wang *et al.*, 2015; Zou *et al.*, 2019). The activation of WRKYs and MYC-2 TFs had been detected in previous studies (Gonzalez-Ibeas *et al.*, 2012). Even though these classes of TFs were not widely up-regulated in this work, other TFs implied in phytohormone synthesis and signaling were strongly over-expressed in the resistant RIL-10-3. In fact, three GCs (GCs13, 14 and 17) whose expression profiles statistically differed for the interaction term between genotype and treatment were enriched in `plant hormone signal transduction´ pathways. The activation of these kind of TFs has also been reported in other cucurbits inoculated with viruses (Amoroso *et al.*, 2021; Li *et al.*, 2017; Lou *et al.*, 2020; Sáez *et al.*, 2022a; Sun *et al.*, 2019). Moreover, the up-regulation of phytohormone biosynthesis and signaling is a common resistance mechanism against viruses. Phytohormones such as SA, ET or JA can prime long distance leaf-to-leaf signaling resistance pathways and also activate resistance genes (Huang *et al.*, 2016). In addition to the previously named TFs there were other upregulated DEGs involved in phytohormones pathways, including two genes coding stem-specific proteins TSJT1 and a gene coding a glycosyltranseferase, that are both implied in SAR (Spagnolo *et al.*, 2014; Mohnike *et al.*, 2021). Phytohormone biosynthesis was also activated in other cucurbits inoculated with viruses (Amoroso *et al.*, 2021; Li *et al.*, 2017; Lou *et al.*, 2020; Sáez

et al., 2022a; Sun *et al.*, 2019) and had previously reported in TGR-1551 plants inoculated with WMV (Gonzalez-Ibeas *et al.*, 2012).

MAPKs and TFs involved in plant defense functions can also interact with the ubiquitin proteasome system. The terms `proteasome´ and `ubiquitin mediated proteolysis´ were enriched in five GCs (GCs 1, 7, 14, 17, 19). Moreover, 27 and 9 DEGs related with the ubiquitination process were up- and down-regulated, respectively. This strong deregulation of the expression of genes involved in proteolysis processes could be directly related to the degradation of viral components. The E3 ubiquitin-protein ligase was repressed in a susceptible accession *of Luffa cilyndrica* inoculated with CMV (Lou *et al.*, 2020) and an altered expression of genes involved in ubiquitination has also been reported in both resistant and susceptible melon accessions inoculated with TolCNDV (Sáez *et al.*, 2022a).

On the other hand, most of the recessive resistances to RNA plant viruses in general, and to potyvirus in particular are related to mutations in the eukaryotic translation initiation factors eIF4E/G or their isoforms eIFiso4E/G (Sanfaçon, 2015). The eIF4E/G complex mediates the recruitment of ribosomes, and it is commonly used by viruses to initiate the translation process of their RNA genome, that is why natural mutations in their coding sequences lead to a broad-spectrum resistance by loss of function (Kachroo *et al.*, 2017; Machado *et al.*, 2017). However, the silencing of the eIF4E in melon did not lead to resistance against WMV, which could mean either that this virus can use the isoform eIFiso4E or that it does not need these factors to complete its life cycle (Rodríguez-Hernández *et al.*, 2012). Other translation initiation factors such as eIF4G or eIF4A-like helicases (DEAD-box ATP-dependent RNA helicases) are also frequently used by viruses and they constitute an alternative for antiviral strategies (Sanfaçon, 2015). The main function of RNA helicases is to perform RNA duplex unwinding and remodel RNA protein complex in cell. DEAD-box helicases also affect virus replication and viral translation acting as viral RNA sensors or as effectors blocking viral RNA replication (Del Toro Duany *et al.*, 2015; Kachroo *et al.*, 2017; Li *et al.*, 2016; Noueiry *et al.*, 2000; Ori *et al.*, 2017). For example, the plant DDX17-like RH30 DEAD-box helicase is a strong restriction factor against tombusviruses and related plant viruses (Wu & Nagy, 2019). Additionally, several DEAD-box helicases have also been described as interfering in ToLCNDV replication (Pandey *et al.*, 2019). Four DEAD-box ATP-dependent RNA helicases (MELO3C023052, MELO3C009973, MELO3C017907, MELO3C006599) were over-expressed in the resistant RIL 10-3 compared to the susceptible cultivar BO. Up-regulation of DEAD-box helicases had previously been

detected in other resistant cucurbits when inoculated with potyvirus (Amoroso *et al.*, 2021) and mutations in DEAD-box helicases had been related to the inhibition of viral translation (Noueiry *et al.*, 2000). SNPs with a high predicted functional impact were detected in the four up-regulated DEAD-box helicases. Thus, the over-expression of these mutated DEAD-box helicases in the resistant genotype could be related to the inhibition of the viral replication.

4. Conclusions

This work presents the first RNA-seq study of the transcriptomic changes due to WMV infection in cucurbits, concretely in melon. In total, 616 genes were detected as differentially expressed between the resistant and the susceptible genotype. This analysis has allowed the identification of the gen MELO3C021395, which coded a mediator of RNA polymerase II transcription subunit 33A (MED33A), as a candidate gen conferring resistance against WMV. Moreover, the WGCNA performed on the global gene expression dataset detected 19 GCs, of which 7 were differentially expressed. The inoculation of WMV triggered a huge transcriptomic remodeling in the resistant genotype, including genes located within and out of the previously described resistant QTLs. The early response turned out to be comprehensive, including genes involved in plant-pathogen interaction, plant hormone signal transduction, the MAPK signaling pathway or ubiquitin mediated proteolysis. As a consequence of the activation of these mechanisms, the photosynthetic pathway, as well as the synthesis of basal metabolites, was altered in the resistant genotype. These results will be useful to better understand the mechanisms underlaying the resistance response against WMV in melon.

5. Methods

5.1 Biological materials

The WMV resistant RIL 10-3, derived from the initial cross between the resistant accession TGR-1551 (*C. melo*, acidulus group) and the susceptible Spanish cultivar `Bola de Oro´ (BO) (*C. melo*, ibericus group), and its parental line BO were used as plant materials in this study. This resistant RIL-10-3 carried the regions on chromosomes 11 and 5 previously linked to the resistance to WMV. Moreover, RIL-10-3 and BO shared approximately a 50% of their genome, which is useful to reduce the background noise in an RNA-seq assay. Seeds of both lines were germinated following the protocol described in (Sáez *et al.*, 2022a) to ensure a homogeneous germination. The plants were grown in a growth chamber under controlled conditions of 27°C, 16h/8h of light and darkness, respectively, and watering as needed.

The WMV virus used in this assay was originally collected in melon infected plants in Huerta de Vera fields (Valencia, Spain). This isolate, WMV-Vera, has been characterized and it belongs to the `emerging´ group (Aragonés *et al.*, 2019) (GenBank: MH469650.1).

5.2 Sampling design, inoculation and symptoms assessment

At the two true-leaves stage, 24 seedlings of both susceptible and resistant genotypes were mechanically inoculated with isolate WMV-Vera. The inoculum was prepared by crushing symptomatic leaves of infected melon plants. Inoculation was performed by rubbing the leaves with a swab with the inoculum, an inoculation buffer and carborundum as described by (López *et al.*, 2015). The same number of plants were mock-inoculated following the same protocol but using only the inoculation buffer and carborundum. Plants were cultivated for 30 days after mechanical inoculation (dpi) under controlled conditions previously described. Four different plants per treatment (susceptible/resistant genotype x virus/mock inoculated treatment) were sampled at 0, 3, 7, 10 and 15 days after inoculation (dpi). For each plant, a leaf disc was collected in a 1.5 ml microtube tube from each of the expanded leaves but the inoculated leaves. Samples were immediately frozen in liquid nitrogen and stored at -80ºC. All the plants were maintained until 30 dpi to phenotype the symptoms.

Additionally, at 0 dpi (two true-leaves stage) (i.e., before inoculation), all expanded leaves of three healthy seedlings of each genotype were collected and used as the control treatment, maintaining those plants alive with their apex intact. Sampling was performed as previously described.

Symptoms of WMV infection were assessed at every sampling point (3, 7, 10 and 15 dpi), with a scale from 0 (no symptoms) to 4 (severe mosaic and leaf distortion). Virus infection also was assessed by RT-qPCR following the method described by (Sáez *et al.*, 2022a) using the primers WMV-CE-170: TGTTGCTTCATGGAAGATTGGT and WMV-CE-171: AAAATTGTGCCATCAGGTGCTA

5.3 RNA extraction and library preparation

For the transcriptomic analysis, three biological replicates at 0 and 3 dpi were selected (i.e. 18 samples = 3 replicates x 2 genotypes at 0 pdi + 3 replicates x 2 genotypes x 2 treatments at 3 dpi). In all 3dpi samples presence or absence of WMV was confirmed by RT-qPCR.

Total RNA was extracted using 700 µL of Extrazol® EM30 (Blirt DNA, Gdansk, Poland) according to kit´s specifications. RNA integrity was checked by 1.5% agarose gel electrophoresis, and purity and quantity were measured using a NanoDrop 1000 (Thermo Scientific, Waltham, MA, USA). Total RNA (2 µg) of the selected 18 plants was sent to Macrogen Inc (Seoul, Republic of Korea) for cDNA library construction. RNA integrity was measured using an Agilent Technologies 2100 Bioanalyzer with an RNA Integrity Number (RIN) value ≥ 7 and an RNA ratio ≥1. Approximately 1 µl of total RNA was used to construct the RNA-seq libraries using the TruSeq Stranded mRNA LT Sample Prep Kit (Illumina) by following manufacturer´s instructions. Finally, libraries were sequenced (paired-end 150 bp) using a NovaSeq6000 Sequencing System (Illumina, CA, USA) and producing more than 40 million reads per sample.

5.4 RNA-seq analysis and differentially expressed genes analysis

Quality of raw sequences was checked using FastQC v0.11.9 (Andrews, 2010). Sequences were processed using Trimmomatic 0.38.0 (Bolger *et al.*, 2014), to remove adapters and low-quality reads. Quality of the trimmed and clean reads was checked again with FASTQC. Trimmed reads were mapped using STAR v. 2.02.01 (Dobin *et al.*, 2013) to the latest version of the melon reference genome (v.4.0) (Castanera *et al.*, 2020) (available at www.melonomics.net) and the number of reads assigned to each gene was quantified using RSEM v. 1.3.1 (Li & Dewey, 2011).

Two kinds of analysis were then performed. First, genes sharing similar expression profiles across all samples were obtained by performing a weighted gene co-expression network analysis, using the R package `WGCNA´ v.1.69 (Langfelder & Horvath, 2008). To test for statistical differences due to the effects of genotype and treatment, a generalized linear model using the cluster's eigengenes was performed. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis (Kanehisa & Goto, 2000) of genes that were significantly enriched at adjusted p-value < 0.01 with each gene cluster was performed using clusterProfiler v.4.4.1 (Wu *et al.*, 2021). Secondly, differentially expressed genes (DEGs) were detected by using DESeq2 v.1.26.0 (Love *et al.*, 2014) and edgeR v.3.38.1 (Robinson *et al.*, 2010) R packages. Number of counts were normalized using the trimmed mean algorithm (TMM) as implemented in edgeR to correct for library sizes. In both cases, a linear model considering the effect of genotype (resistant (RIL-10-3) or susceptible (BO)), the effect of treatment (mock or WMV-inoculated) and its interaction at 3dpi was used. DEGs showing a significant interaction term after correction for multiple testing in DESeq2 and edgeR (adjusted p-value < 0.05) with a log2FC ≥ 1 or log2FC≤-

1 (log2 fold change) were considered for subsequent enrichment analysis. A consensus list of DEGs between both algorithms was obtained. DEGs between genotypes at 0 dpi were also obtained and used to check that the DEGs detected at 3 dpi were not due to genotypic differences. DEGs between resistant and susceptible genotypes were identified at 0 dpi and it was checked that those detected DEGs were not included within the list of DEGs detected for the interaction term at 3 dpi. Finally, the Cucurbits Genomics Database (CuGenDB, http://cucurbitgenomics.org/) was used to determine the enriched biological functions and processes related to the DEGs detected.

5.5. Transcript-Based Single Nucleotide Polymorphism Identification

Additionally, to identify mutations that could be related to the defense response, single nucleotide polymorphisms (SNPs) in the sequenced transcripts were detected. Clean reads were aligned to the reference melon genome by using bowtie2 v.2.3.4. (Langmead & Salzberg, 2012) and SNP calling was carried out with Freebayes v.1.3.4 (Garrison & Marth, 2012a). Variant annotation and its predicted effect on the transcript were assayed using SNPEff v.5.0e (Cingolani *et al.*, 2012).

To validate the observed genotype related to the gene *CmVps4*, a fragment of this gene was amplified by PCR in two TGR-1551, BO and RIL-10-3 cDNA samples. The corresponding PCR product was purified using the EXTRACT-ME DNA CLEAN-UP KIT (BLIRT S.A. Gdansk, Poland) and paired-end sequenced by Sanger method (Secuenciación de ADN y análisis de la expression génica, Instituto de Biología Molecular y Celular de Plantas (IBMCP), Valencia, Spain) using the primers *CmVps4P30R-F*: TCCGTCGTTCGCTTTAGTCT and *CmVPs4P30R-R*: AGTTGCAACAGCTGCATCAC.

5.5 Validation of differentially expressed genes by RT-qPCR

To validate the RNA-seq data, the expression patterns of 6 candidate genes, putatively associated to WMV resistance in TGR-1551, were evaluated by quantitative real time PCR (qRT-PCR). Four biological and two technical replicates of both mock and WMV-inoculated plants were evaluated at 3 dpi. Total RNA (1 µg) was treated with PerfeCTa® DNase I (RNAse-free) (Quanta Biosciences, Gaithersburg, MD, United States) and reversed transcribed with RevertAid™ First Strand cDNA Synthesis Kit (ThermoFisher Scientific) and oligo (dT)20 as reverse primer. Quantitative PCR were carried out on a LightCycler480 Real-Time PCR system (Roche Applied Science, IN, USA), using the FastStart Essential DNA Green Master (Roche Molecular Systems, Rotkreuz, Switzerland) and 1.5 µl of cDNA as

template. Primers sequences are listed in Additional table 2. Melon cyclophilin (*CmCYP7*) (Gonzalez-Ibeas *et al.*, 2007) was used as reference gene. The efficiency of the primers was studied from the slope of the linear correlation between Ct and each dilution ($E = 10^{(-1/slope)}$). To ensure specific product amplification and to avoid quantification of primer-dimers, melting curve analysis (60-95 °C) at the reaction end-point and no-template controls were used. The relative quantitative expression of each gene was calculated with the ΔCt method.

A full linear model including genotype and treatment as fixed factors the double interaction (ΔCt ~ genotype + treatment + genotype*treatment) was used to study the observed differences in ΔCt values using the R package `stats´ v.4.2.2. Normal distribution of the data was analyzed with a Shapiro-Wilk normality test, included in the R package `rstatix´ v.0.7.2 (Kassambara, 2023). As some factors combinations did not follow a normal distribution, robust two-way ANOVAs were calculated using the R package `WRS2´ (Mair & Wilcox, 2020). Finally, for the significant ANOVA test a least significant different (LSD) test, included in the R package `agricolae´ v.1.3.5 (de Mendiburu, 2021), was performed.

List of abbreviations

ABA, abscisic acid; AUX, auxins; Bg7S, basic 7S globulin-like protein; bHLH, basic helix-loop-helix; BO, `Bola de Oro´; BR, brassinosteroids; bZIP, basic leucine zipper; C4H, cinnamate 4-hydroxylase; CDPK, calcium-dependent protein kinase; chr, chromosome; CK, cytokinin; CMV, cucumber mosaic virus; DEGs, differentially expressed genes; dpi, days post-inoculation; ERFs, ethylene responsive transcription factors; ET, ethylene; ETI, effector-triggered immunity; GBS, genotyping by sequencing; GCs, gene clusters; HSP, heat shock proteins; JA, jasmonate; KEGG, Kyoto Encyclopedia of Genes and Genomes; log2FC, log2 fold change; MAPK, mitogen-activated protein kinase; MED33A, RNA polymerase II transcription subunit 33A; MYB, myeloblastosis related; NAC, no apical meristem; NGS, new generation sequencing; NHP, N-hydroxy-pipecolic acid; PA, piperonylic acid; PAL1, phenylalanine-ammonia lyase 1; PAL2, phenylalanine-ammonia lyase 12; PAMPs, pathogen associated molecular patterns; PPP, phenylpropanoid pathway; PR, pathogenesis related; QTLs, quantitative trait loci; REF4, reduced epidermal fluorescence 4; RFR1, REF4-related 1; RILs, recombinant inbred lines; RIN, RNA Integrity Number; RLKs, receptor-like kinases; RNAPII, RNA polymerase II; ROS, reactive oxygen species; RPKs, receptor protein-kinases; SA, salicylic acid; SAR, systemic acquired resistance; SNP, single nucleotide polymorphism; TCA, citric acid cycle; TF, transcription factor; TMM, trimmed mean algorithm; ToLCNDV, Tomato leaf curl New Delhi virus; Trxs, thioredoxin-like protein; UPS, ubiquitin proteasome

system; WGCNA, weighted gene co-expression network analysis; WMV, Watermelon mosaic virus; XEG, xyloglucan-specific endo-β-1,4-glucanase; ZYMV, Zucchini yellow mosaic virus

Competing interests

The authors declare that they have no competing interests

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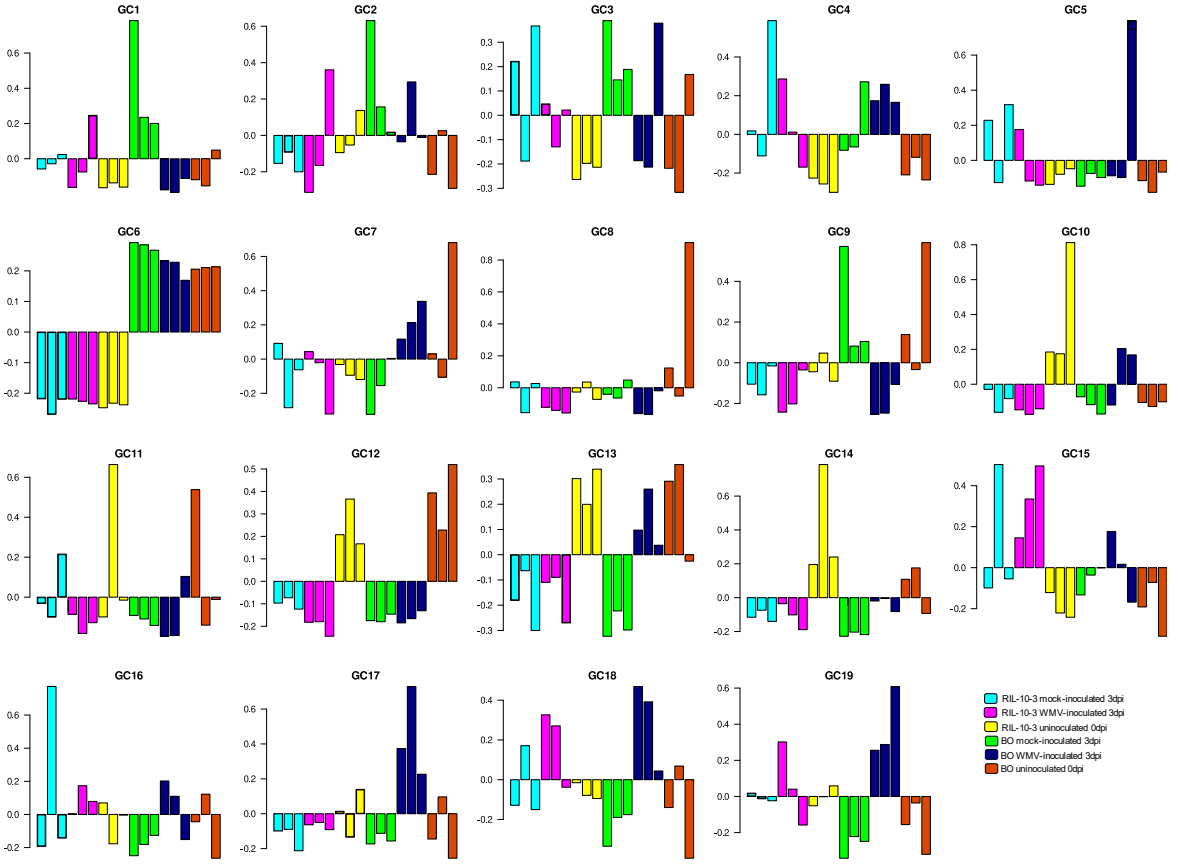
Authors´contributions

Conceptualization, A.P.-d.-C., M.L.G.G and B.P.; methodology, A.P.-d.-C. M.L.M. G.Y. and J.M.P..; software, M.L.-M, J.M.-P. and G.Y.; investigation, M.L.G.-G., B.P., M.L.-M. and A.P.-d.-C.; writing—original draft preparation, review and editing, M.L.-M., A.P.-d.-C., J. M. -P., G.Y., M.L.G.-G. and B.P.; funding acquisition, M.L.G.-G., A.P.-d.-C. and B.P. All authors have read and agreed to the published version of the manuscript.

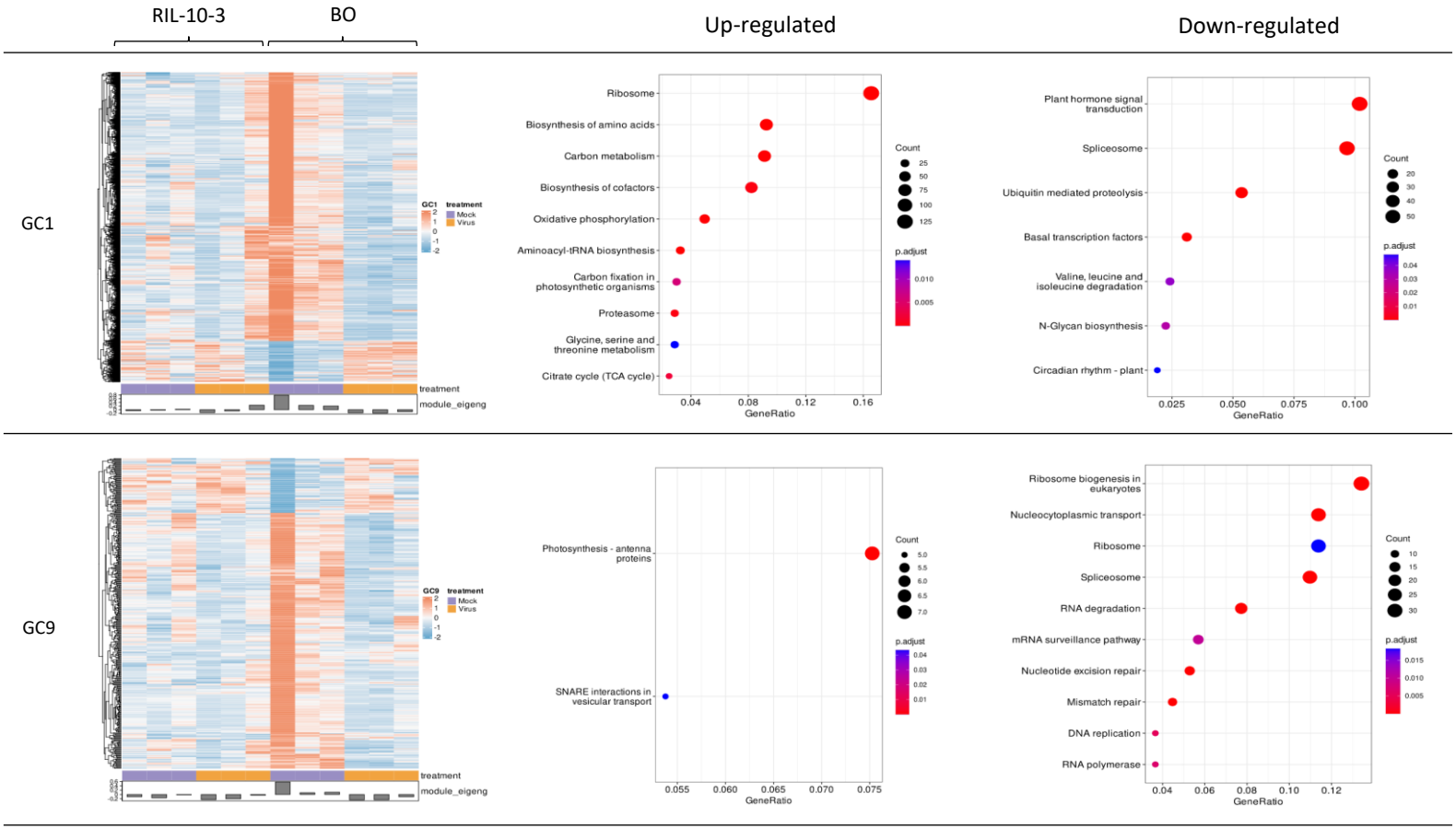
Supplementary materials

Supplementary materials can be found at: https://drive.google.com/drive/folders/1NVFWpHHktFK0Du7aaoZxaHrX-WlkS1U2?usp=sharing

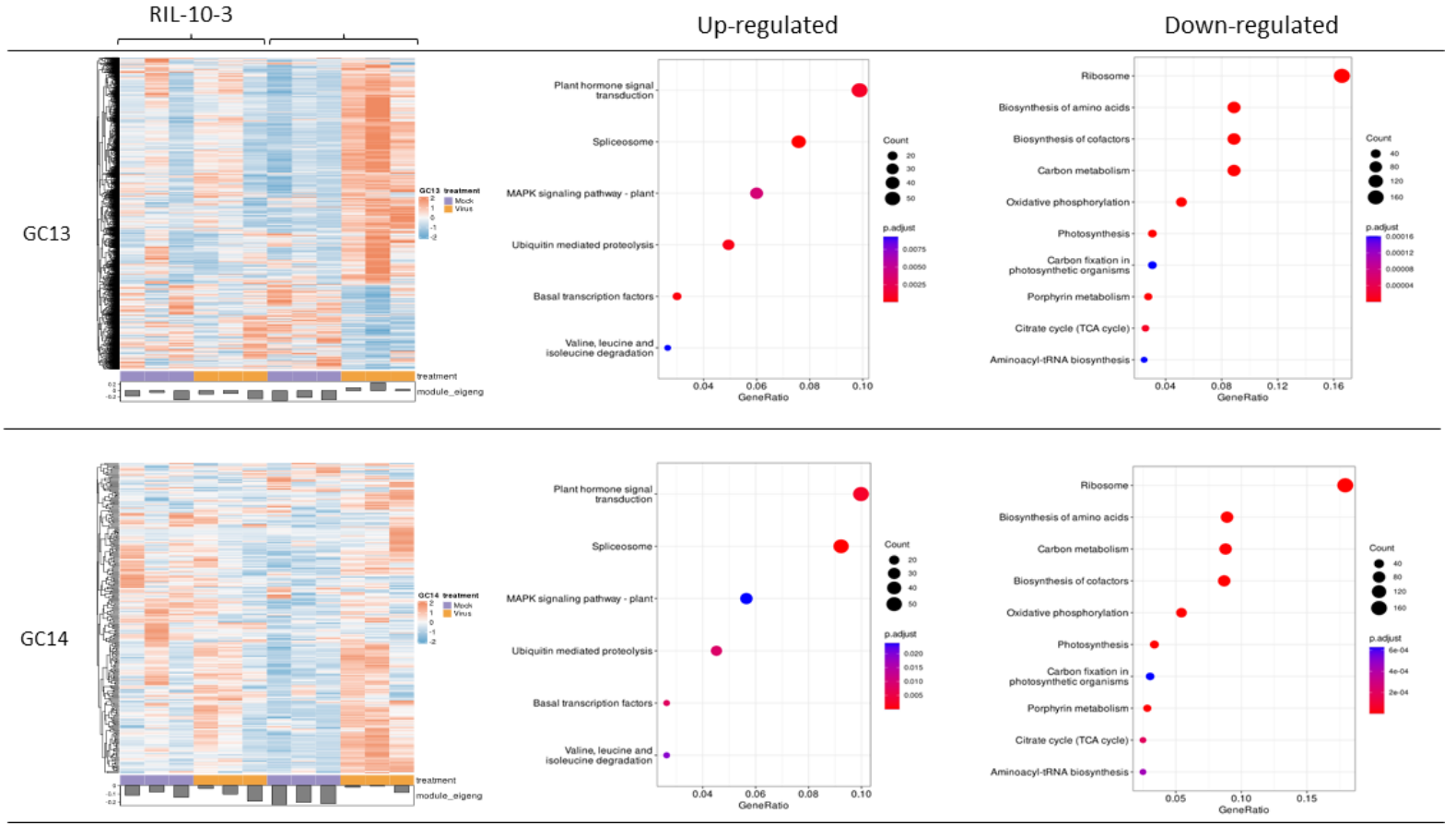
Additional Figure 1. Module eigengenes across samples for the gene clusters detected by WGCNA



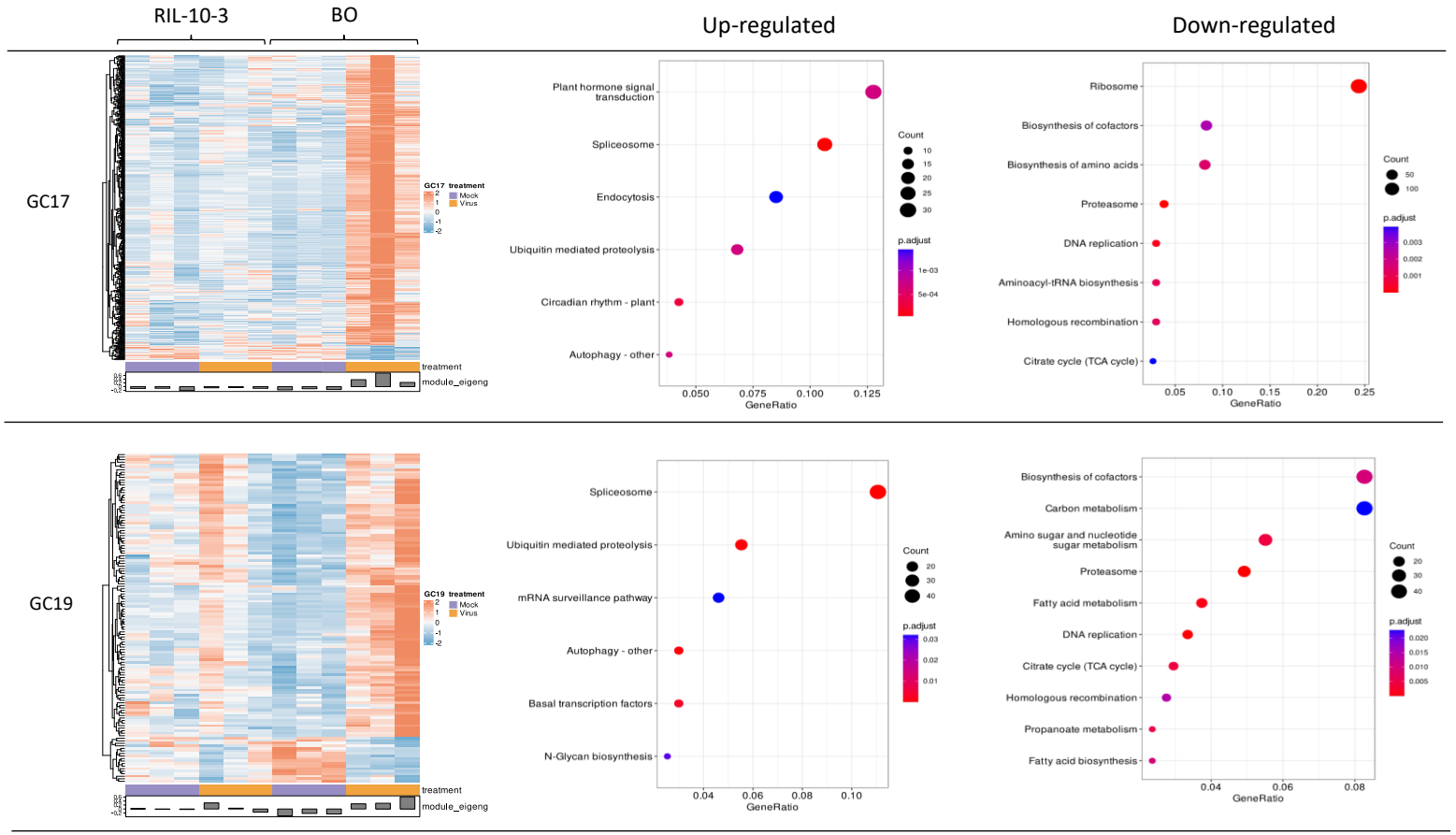
Additional Figure 2. Heatmap and KEGG enrichment profile of up- and down-represented genes within each cluster that had been identified as differentially expressed by WGCNA.



Additional Figure 2. Continuation



Additional Figure 2. Continuation



Additional Table 1. List of differentially expressed genes (DEGs) detected between RIL-10-3 and BO for the interaction termn genotype x treatment. The chromosome were the corresponding gene is located, its start and end position in the melon genome (v.4.0), log fold change (logFC), log counts per million reads (logCPM), F value, P-value and false discovery rate (FDR), as well as its annotation in the reference genome are indicated.

Due to its extent, the additional table 1 can be found in the following link: https://drive.google.com/drive/folders/1NVFWpHHktFK0Du7aaoZxaHrX-WlkS1U2?usp=sharing

Additional Table 2. List of primers used por RT-qPCR validation of the RNA-seq data.

Amplificated gene		F primer sequence		R primer sequence
MELO3C021413	MELO3C021413-F	TTATATGGGCCTCCTGGAAC	MELO3C021413-R	CGAGACAAGGTCCGAAGAAG
MELO3C004433	MELO3C004433-2F	TTTGAAGTCGTAATGTGAAAACT	MELO3C004433-2R	ACGGAAAAGGCTCACTTCAC
MELO3C004448	MELO3C004448-F	GCTTCCTGCACATTTTCCTC	MELO3C004448-R	ACAGTAAGGCAGCTGGAAGC
MELO3C004204	MELO3C004204-F	GTCTTCAAGCCCAGCAACTC	MELO3C004204-R	GCAACCATGGAAGGAAGATG
MELO3C021395	mediator-Fbueno2	ACACAGCATGAGCAGAG	mediator-Rbueno	CAGCAACCATTCTTTGTGGG
MELO3C021406	MELO3C021406-F	ACGAACGGTGTTGTTTTTCC	MELO3C021406-R	TAAAATACTCCGGCGACAGC

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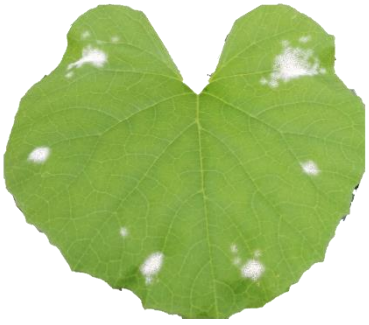
Chapter 4.

Advanced Genetic Studies on Powdery Mildew Resistance in TGR-1551



Chapter 4. Advanced genetic studies on powdery mildew resistance in TGR-1551

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Abstract

Cucurbits powdery mildew (CPM) is one of the main limiting factors of melon cultivation worldwide. Resistance to races 1, 2, and 5 has been reported in the African accession TGR-1551, whose resistance is controlled by a dominant–recessive epistasis. The dominant and recessive quantitative trail loci (QTL) have previously been located in chromosomes 5 and 12, respectively. We used several densely genotyped BC_3 families derived from the cross between TGR-1551 and the susceptible cultivar 'Bola de Oro' to finely map these resistance regions. The further phenotyping and genotyping of the selected BC_5, BC_5S_1, BC_5S_2, BC_4S_1, BC_4xPS, and (BC_4xPS) S_1 offspring allowed for the narrowing of the candidate intervals to a 250 and 381 kb region in chromosomes 5 and 12, respectively. Moreover, the temperature effect over the resistance provided by the dominant gene has been confirmed. High resolution melting markers (HRM) were tightly linked to both resistance regions and will be useful in marker-assisted selection programs. Candidate R genes with variants between parents that caused a potential modifier impact on the protein function were identified within both intervals. These candidate genes provide targets for future functional analyses to better understand the resistance to powdery mildew in melons.

Keywords: powdery mildew; molecular markers; dominant–recessive epistasis; marker-assisted selection; TIR-NBS-LRR

1. Introduction

Melons (*Cucumis melo* L.) constitute an economically important vegetable crop. In 2019, their world production reached 27,5 million tons (http://faostat3.fao.org (accessed on 1 June 2022)). Among the most important diseases affecting this crop, cucurbit powdery mildew (CPM), a fungal disease occurring in both field and greenhouse conditions worldwide, reduces its productivity. The occurrence of CPM early in the growing season can lead to a massive reduction in photosynthetic capacity, with negative impacts on plant growth and development, as well as fruit quality (Cohen *et al.*, 2004; Lebeda *et al.*, 2007, 2009). Two ectoparasites, *Golovinomyces orontii* (*Go*) and *Podosphaera xanthii* (*Px*), are economically important and distributed worldwide (Braun, 2012), as they are both the two most commonly reported causal agents of CPM (Křístková *et al.,* 2009; Lebeda *et al.*, 2021).

Spatiotemporal changes in the geographic distribution of both *Go* and *Px* have been observed during the last three decades (Křístková *et al.,* 2009; Lebeda *et al.*, 2007, 2009). Significant variations in virulence, expressed by numerous pathotypes

and/or races and their spatiotemporal fluctuations, have been described for both species (Bardin *et al.*, 1999; Cohen *et al.*, 2004; Hong *et al.*, 2018; Lebeda & Sedláková, 2006; Lebeda *et al.*, 2007, 2008, 2009). De Miccolis Angelini *et al.* (2019) emphasized that *Px* has great potential to evolve new, better-adapted genotypes that can overcome the currently used resistance genes and the efficacy of modern fungicides. Variability in *Px* virulence, recorded as different physiological races, was first observed in the U.S. in 1938, when race 2 appeared shortly after the release of race 1-resistant 'PMR 45' produced in Imperial Valley, California (McCreight *et al.*, 2018). Since then, many physiological races have been identified according to the reactions after infection of a set of differential melon lines, and several cultivars with resistance to some of those races have been described.

To date, several genes and QTLs associated with resistance to CPM have been mapped in different melon populations derived from several genetic sources, including *Pm-x* (Périn *et al.*, 2002), *Pm-x1,5* and *Pm-x3* (Fazza *et al.*, 2013), *Pm2F* (Zhang *et al.*, 2013), *QTL (AR5*) (Fukino *et al.*, 2008), and *Pm-Edisto47-2* (Ning *et al.*, 2014) on chromosome 2; *qPx1-4* (Branham *et al.*, 2021) on chromosome 4; *Pm-w* (Pitrat, 1991), *Pm-R* (Yuste-Lisbona *et al.*, 2011a), *Pm-AN* (Wang *et al.*, 2011), *qPx1-5* (Branham *et al.*, 2021), and QTL *PmV-1-Pi124112* (Perchepied *et al.*, 2005) on chromosome 5; *CmPMrs* (Cui *et al.*, 2022a) and *qPx1-10* (Branham *et al.*, 2021) on chromosome 10; *Pm-y* (Périn *et al.*, 2002), QTL *PmXII-1-Pi124112* (Perchepied *et al.*, 2005), *QTL (AR5)* (Fukino *et al.*, 2008), *Pm-Edisto47-1* (Ning *et al.*, 2014), *CmPMR1* (Cui *et al.*, 2022a), *qPx1-12* (Branham *et al.*, 2021), *qCmPMR-12* (Cao *et al.*, 2021), and *BPm12.1* (Li *et al.*, 2017) on chromosome 12; and *Pm-1* (Teixeira *et al.*, 2008) on LGIX. Most of these resistances are race-specific and controlled by one or two dominant genes. Epistatic effects have been described between *CmPMrs* and *CmPMR1* QTLs (Cui *et al.*, 2022a), as well as between *qPx1-5* and *qPx1-12* (Branham *et al.*, 2021). In addition, several studies reported that Mildew Locus O (MLO) genes act as susceptibility factors in CPM disease and their inactivation of specific MLO genes (knock-out or knock-down) leads to a mediating form of mlo resistance (Hong *et al.*, 2015; Iovieno *et al.*, 2015). Natarajan *et al.* (2016) carried out a comparative analysis of 14 MLO genes and investigated their SNPs and InDels variations, finding associations among them.

Among the resistant cultivars described, the Zimbabwean melon TGR-1551 carries resistance to races 1, 2, and 5 of *Px*. The inheritance of such resistance was studied in a cross of TGR-1551 with the Spanish susceptible cultivar 'Bola de Oro' and the results indicated that it was governed by two independent genes, one dominant and one recessive, whose genetic control corresponded to dominant–recessive

epistasis (Yuste-Lisbona *et al.*, 2010). A QTL analysis carried out on the F_2 generation derived from the initial cross allowed for the identification of one major QTL (*Pm-R*) on chromosome 5 for resistance to the three races (Yuste-Lisbona *et al.*, 2011). A RIL population (F_7:F_8) obtained from that cross was also evaluated for *Px* resistance to races 1, 2, and 5, and QTL analyses carried out by Beraldo-Hoischen *et al.* (2012) confirmed the existence of the *Pm-R* QTL in chromosome 5, associated with the dominant gene. An additional QTL, possibly associated with the recessive gene, was also detected for *Px* resistance. This QTL is located on chromosome 12 and several microsatellite markers (TJ29, CMBR111, and CMBR150) were described as being associated with resistance (Beraldo-Hoischen *et al.*, 2012). Two QTL associated with resistance to Px had been previously identified on chromosome 12. One of them is the QTL *PmXII.1* (Perchepied *et al.*, 2005), associated with the *Pm-y* gene and controlling resistance to races 1, 2, and 5 derived from PI 124112. The direct association of *PmXII.1* with the minor QTL reported in TGR-1551 was not possible due to the lack of common molecular markers between both genetic maps (Beraldo-Hoischen *et al.*, 2012). The other QTL was identified by (Fukino *et al.* (2008) and associated with one of the two dominant genes for resistance to race 1 described previously in PMR 5 (Fukino *et al.*, 2004), as well as with *PmXII.1*. This QTL from Fukino *et al.* (2008) was strongly linked to the microsatellite markers associated with the QTL derived from TGR-1551 (TJ29, CMBR111, and CMBR150) (Beraldo-Hoischen *et al.*, 2012). This candidate interval proposed by Fukino *et al*. (2008) has recently been confirmed by Hong *et al*. (Hong *et al.*, 2022) and the resistance of MR-1 to race 1 has recently been mapped to the same region (Hong *et al.*, 2018; Branham *et al.*, 2021). More recently, Howlader *et al.* (2020) described three SNP markers associated with specific resistance to race 5 of *Px* in chromosome 12, in close proximity to the microsatellite markers previously described by Fukino *et al*. (2008) and Beraldo-Hoischen *et al*. (2012). The recessive gene present in TGR-1551 is unique, since no recessive genes conferring resistance to more than one *Px* race has been previously reported in melons. This gene could confer a durable resistance to powdery mildew, similar to the resistance conferred by *mlo* in barley (Helms Jorgensen, 1992). CPM resistance is an important objective of melon-breeding programs, which implies the identification of molecular markers and possible candidate genes associated with the trait (Varshney *et al.*, 2012).

The objective of this work was to finely map the recessive locus derived from TGR-1551 conferring resistance to *Px* by analyzing the advanced backcross populations segregating only at the target genomic region associated with the recessive gene previously described. In addition, a fine mapping of the genomic area where the dominant resistant gene is located in chromosome 5 has been carried out. The

generations used in this study were constructed from an original BC_3S_1 (200 families) obtained after three successive backcrosses to the susceptible cultivar 'Bola de Oro' (BO).

2.Results

2.1. Selection of BC_3, BC_3S_1, and BC_4 Generations

The resistance to CPM derived from TGR-1551 had previously been described as being controlled by a dominant–recessive epistasis, (Yuste-Lisbona *et al.,* 2010) where a QTL related to the dominant gene was located in chromosome 5 (Beraldo-Hoischen *et al.*, 2012; Yuste-Lisbona *et al.*, 2010, 2011) and another QTL linked to the recessive gene mapped in chromosome 12 (Beraldo-Hoischen *et al*., 2012). 200 BC_3 plants (TGR-1551 x BO derived) were genotyped with three previously existing markers sets (CYSDV1, WMV1 and WMV2) (Pérez-de-Castro *et al.,* 2019, 2020), which mainly covered different regions of the chromosomes 5 and 11. A total number of 52 BC_3 plants carrying different regions introgressed in these chromosomes were selected. An existing panel of 124 SNP markers evenly distributed throughout the genome (Background markers 1) (**Supplementary Table S1**) (Esteras *et al.*, 2013; Leida *et al.*, 2015; Perpiñá *et al.*, 2016; Pérez-de-Castro *et al.*, 2019) were used to genotype these 52 BC_3 plants. For the rest of the genome, a high percentage of the BO genetic background was prioritized (**Table 1**) and 20 BC_3 plants were selected. The selfing progenies and BC_4 offspring (10 to 20 plants) of these 20 plants were genotyped with the set of markers CYSDV1 and CYSDV2 (**Supplementary Table S2**) (Pérez-de-Castro *et al.,*2020). Moreover, those plants were genotyped with the previously developed HRM markers *cysdv63* and *cysdv65* (Pérez-de-Castro *et al.,*2020). These generations were used to finely map the regions associated with resistance.

Table 1. Genotype for the SNP markers distributed evenly throughout the genome and located in chromosomes 5 and 12 and for the SNPs in the panels CYSDV1, CYSV2, WMV1, and WMV2 for the BC_3 plants selected to evaluate their offspring (H: heterozygous; A: homozygous for 'Bola de Oro' allele). The genotype for the set CYSDV2 markers has been inferred from the BC_3S_1 families. The phenotype for the descendants is indicated (SU: susceptible; SE: segregating). Markers for the candidates' intervals are in bod format highlighted.

				Number of BC_3 plant																			
Markers set	**Marker**	**Chr[a]**	**Genomic position (bp)**	**15**	**19**	**34**	**64**	**78**	**95**	**96**	**105**	**139**	**141**	**166**	**24**	**28**	**37**	**88**	**89**	**146**	**148**	**159**	**198**
Background1	*CMPSNP898*	5	256,610	A	A	A	A	A	A	A	H	A	A	H	A	A	A	H	H	n.d	A	A	A
Background1	*CMPSNP387*	5	1,260,621	A	A	A	H	A	A	A	H	A	A	H	A	A	A	A	H	n.d	A	A	A
Background1	*CMPSNP437*	5	1,711,810	A	A	A	H	A	A	A	A	A	H	H	A	A	A	A	H	n.d	H	A	A
Background1	*CMPSNP726*	5	2,373,146	A	H	A	H	A	A	A	A	A	H	H	A	A	A	A	H	n.d	H	A	A
Background1	*SSH9G15*	5	5,781,400	A	A	A	H	A	A	A	A	A	A	H	H	H	A	A	n.d	n.d	H	A	A
CYSDV1	*cysdv10B*	5	6,298,039	A	A	A	H	A	A	A	A	A	A	H	H	H	A	A	A	A	H	H	A
CYSDV1	*cysdv11*	5	9,494,514	A	A	A	H	A	A	A	A	A	A	H	H	H	A	A	A	A	n.d	H	A
Background1	*CMPSNP788*	5	12,024,140	A	A	A	H	A	A	A	A	A	A	H	H	H	A	A	A	A	n.d	H	A
WMV1	*b5wmv2*	5	15,026,076	A	A	A	H	A	A	A	A	A	A	H	H	H	A	A	A	A	H	H	A
CYSDV1	*cysdv14*	5	17,216,441	A	A	A	H	A	A	A	A	A	A	H	H	H	A	A	A	A	n.d	H	A
Background1	*60k41.243*	5	19,612,771	H	H	A	H	A	H	A	H	A	H	H	A	A	A	A	A	A	n.d	H	A
WMV1	*b5wmv3*	5	19,968,717	n.d	A	A	H	A	A	A	A	A	n.d	H	H	n.d	A	A	A	A	n.d	H	A
CYSDV1	*cysdv17*	5	22,544,163	A	A	A	H	A	A	A	A	A	A	H	H	H	A	A	A	A	n.d	H	A
CYSDV1	*cysdv18*	5	24,125,662	A	H	A	H	A	A	A	A	A	A	H	A	H	A	A	A	A	n.d	H	A
CYSDV1	*cysdv19*	5	24,192,280	A	H	A	H	A	A	A	A	A	A	H	A	H	A	A	A	A	n.d	H	A
CYSDV1	*cysdv21*	5	24,427,738	A	H	A	H	A	A	A	A	A	H	H	A	H	A	A	A	H	n.d	H	A
CYSDV1	*cysdv22*	5	24,434,940	A	H	A	H	A	A	A	A	A	H	H	A	H	A	A	A	H	n.d	H	A
CYSDV2	*cysdv40*	5	24,474,795	A	H	A	H	A	A	A	A	A	H	H	A	H	A	A	A	H	n.d	H	A
CYSDV2	*cysdv42*	5	24,608,464	A	H	A	H	A	A	A	A	A	H	H	A	A	A	A	A	H	n.d	H	A

Table 1. continuation

Markers set	Marker	Chr[a]	Genomic position (bp)	Number of BC_3 plant																		
				15	19	34	64	78	95	96	105	139	141	166	24	28	37	88	89	146	148	159	198
CYSDV2	*cysdv43*	5	24,678,113	A	H	A	H	A	A	A	A	A	H	H	A	A	A	A	A	H	n.d	H	A
CYSDV2	*cysdv44*	5	24,704,705	A	H	A	H	A	A	A	A	A	H	H	A	A	A	A	A	H	n.d	H	A
CYSDV2	*cysdv45*	5	24,761,527	A	H	A	H	A	A	A	A	A	H	H	A	A	A	A	A	H	n.d	H	A
CYSDV2	*cysdv46*	5	24,778,081	A	H	A	H	A	A	A	A	A	H	H	A	A	A	A	A	H	n.d	H	A
CYSDV2	*cysdv48*	5	24,842,569	A	H	A	H	A	A	A	A	A	H	H	A	A	A	A	A	H	n.d	H	A
CYSDV2	*cysdv49*	5	24,842,825	A	H	A	H	A	A	A	A	A	H	H	A	A	A	A	A	H	n.d	H	A
WMV2	*SNP25*	5	24,898,321	A	H	A	H	A	A	A	A	A	H	H	A	A	A	A	A	H	A	H	A
WMV2	*SNP26*	5	25,045,765	H	H	A	H	A	A	A	A	A	H	H	A	A	A	A	A	H	n.d	H	A
CYSDV2	*cysdv50*	5	25,052,002	H	H	A	H	A	A	A	A	A	H	H	A	A	A	A	A	H	n.d	H	A
CYSDV2	*cysdv51*	5	25,132,216	H	H	A	H	A	H	A	A	A	H	H	A	A	A	A	A	H	n.d	H	A
WMV1	*b5wmv4*	5	25,132,292	H	H	A	H	A	H	A	A	A	H	H	A	A	A	A	A	H	n.d	H	A
WMV1	*b5wmv4A*	5	25,144,133	H	H	A	H	A	H	A	A	A	H	H	A	A	A	A	A	H	n.d	H	A
CYSDV2	*cysdv53*	5	25,144,085	H	H	A	H	A	H	A	A	A	H	H	A	A	A	A	A	H	n.d	H	A
CYSDV2	*cysdv54*	5	25,210,212	H	H	A	H	A	H	A	A	A	H	H	A	A	A	A	A	H	n.d	H	A
CYSDV2	*cysdv55*	5	25,210,573	H	H	A	H	A	H	A	A	A	H	H	A	A	A	A	A	H	n.d	H	A
CYSDV2	*cysdv56*	5	25,233,221	H	H	A	H	A	H	A	A	A	H	H	A	A	A	A	A	H	n.d	H	A
CYSDV2	*cysdv57*	5	25,341,873	H	H	A	H	A	H	A	A	A	H	H	A	A	A	A	A	H	n.d	H	A
CYSDV2	*cysdv58*	5	25,356,079	H	H	A	H	A	H	A	A	A	H	H	A	A	A	A	A	H	n.d	H	A
CYSDV2	*cysdv59*	5	25,435,244	H	H	A	H	A	H	A	A	A	H	H	A	A	A	A	A	H	n.d	H	A
WMV1	*b5wmv5*	5	25,694,699	H	H	A	H	A	H	A	A	A	H	H	A	A	A	A	A	H	n.d	H	A
CYSDV2	*cysdv60*	5	25,744,140	H	H	A	H	A	H	A	A	A	H	H	A	A	A	A	A	H	n.d	H	A
CYSDV2	*cysdv61*	5	25,756,801	H	H	A	H	A	H	A	A	A	H	H	A	A	A	A	A	H	n.d	H	A

Table 1. Continuation

Markers set	Marker	Chr[a]	Genomic position (bp)	Number of BC_3 plant																			
				15	19	34	64	78	95	96	105	139	141	166	24	28	37	88	89	146	148	159	198
CYSDV2	*cysdv61*	5	25,772,357	H	H	A	H	A	H	A	A	A	H	H	A	A	A	A	A	H	n.d	H	A
CYSDV2	*cysdv62*	5	25,776,015	H	H	A	H	A	H	A	A	A	H	H	A	A	A	A	A	H	n.d	H	A
CYSDV2	***cysdv63***	**5**	**25,782,654**	**H**	**H**	**A**	**H**	**A**	**H**	**A**	**A**	**A**	**H**	**H**	**A**	**A**	**A**	**A**	**A**	**H**	n.d	**H**	**A**
Background1	***CMPSNP464***	**5**	**26,405,006**	**H**	**H**	**A**	**H**	**A**	**H**	**A**	**H**	**A**	**A**	**H**	**A**	**A**	**A**	**A**	**A**	**n.d**	**A**	**H**	**H**
CYSDV2	***cysdv65***	**5**	**26,408,895**	**H**	**H**	**A**	**H**	**A**	**H**	**A**	**H**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	n.d	**H**	**H**
CYSDV2	*cysdv69*	5	26,467,357	H	H	A	H	A	H	A	H	A	A	A	A	A	A	A	A	A	n.d	H	H
CYSDV1	*cysdv24*	5	26,547,204	H	H	A	A	A	H	A	H	A	A	A	A	A	A	A	A	A	n.d	H	H
WMV1	*b5wmv7*	5	26,592,732	H	H	A	A	A	H	A	H	A	A	A	A	A	A	A	A	A	n.d	H	H
WMV1	*b5wmv7B*	5	26,592,732	H	H	A	A	A	H	A	H	A	A	A	A	A	A	A	A	A	n.d	H	H
CYSDV1	*cysdv23*	5	26,752,698	H	H	A	A	A	H	A	H	A	A	A	A	A	A	A	A	A	n.d	H	H
CYSDV1	*cysdv25*	5	26,769,546	H	H	A	A	A	H	A	H	A	A	A	A	A	A	A	A	A	n.d	H	H
CYSDV1	*cysdv26*	5	26,940,168	H	H	A	A	A	H	A	H	A	A	A	A	A	A	A	A	A	n.d	H	H
CYSDV1	*cysdv27*	5	26,957,159	H	H	A	A	A	H	A	H	A	A	A	A	A	A	A	A	A	n.d	H	H
WMV1	*b5wmv8*	5	26,963,176	H	H	A	A	A	H	A	H	A	A	A	A	A	A	A	A	A	n.d	H	H
CYSDV1	*cysdv28*	5	27,118,062	H	H	A	A	A	H	A	H	A	A	A	A	A	A	A	A	A	n.d	H	H
WMV1	*b2wmv9*	5	27,273,256	H	H	A	A	A	H	A	H	A	A	A	A	A	A	A	A	A	n.d	H	H
WMV1	*b2wmv10*	5	27,464,146	H	H	A	A	A	H	A	H	A	A	A	A	A	A	A	A	A	n.d	H	H
WMV2	*SNP29*	5	27,538,308	H	H	A	A	A	H	A	H	A	A	A	A	A	A	A	A	A	n.d	H	H
WMV1	*b5wmv11*	5	27,570,154	H	H	A	A	A	H	A	H	A	A	A	A	A	A	A	A	A	n.d	H	H
CYSDV1	*cysdv30B*	5	27,570,488	H	H	A	A	A	H	A	H	A	A	A	A	A	A	A	A	A	n.d	H	H
Background1	*AI_13-H12*	5	28,039,739	H	H	A	A	H	A	A	H	A	A	A	A	A	A	A	A	A	A	H	H

Table 1. Continuation

Markers set	Marker	Chr[a]	Genomic position (bp)	Number of BC_3 plant																			
				15	19	34	64	78	95	96	105	139	141	166	24	28	37	88	89	146	148	159	198
Background1	*CMPSNP385*	12	344,819	A	A	A	A	A	H	A	A	A	A	A	A	A	A	A	H	n.d	A	A	A
Background1	*CMPSNP310*	12	5,032,799	A	A	A	A	A	H	A	A	A	A	A	A	A	A	A	A	n.d	A	A	H
Background1	*AI_35-A08*	12	12,750,025	A	A	A	A	H	A	A	A	A	A	A	A	A	A	A	A	n.d	A	A	H
Background1	*ai09g07*	12	16,532,245	A	A	A	A	H	A	A	A	A	A	A	A	A	A	A	A	n.d	A	A	H
Background1	***CMPSNP285***	**12**	**20,421,309**	**A**	**A**	**H**	**A**	**H**	**H**	**A**	**A**	**H**	**H**	**A**	**A**	**A**	**A**	**H**	**A**	**n.d**	**A**	**A**	**H**
Background1/2	***CMPSNP361***	**12**	**23,000,406**	**A**	**A**	**H**	**A**	**A**	**H**	**H**	**H**	**H**	**H**	**A**	**A**	**A**	**A**	**H**	**H**	**A**	**H**	**A**	**A**
Background1	*CMPSNP5*	12	24,246,762	A	A	H	A	A	H	H	A	A	H	A	H	A	A	A	H	n.d	H	A	A
Background1	*fr14f22*	12	25,050,570	H	A	A	A	A	H	H	A	A	H	A	H	A	A	A	H	n.d	A	A	A
Background1	*P02.03*	12	25,661,792	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	n.d	A	A	A
Phenotype				SE	SE	SE	SE	SE	SE	SE	SE	SE	SE	SE	SU	SU	SU	SU	SU	SU	SU	SE	SU

[a] : chromosome

2.2. Narrowing of the Candidate Region on Chromosome 5

The 20 BC_3S_1 families were phenotyped for the resistance against races 1, 2, and 5 of *Px* (**Table 1**). The response of the offspring of those plants not carrying a TGR-1551 introgression in chromosome 12 depended on the genotype in chromosome 5. Segregation for the resistance was observed for those families with parents heterozygous for the region between markers *cysdv63* (chr5: 25,782,654 bp) and *CMPSNP464* (chr5: 26,405,006 bp) (BC_3 plants 19, 64, 159, and 166) (**Table 1**). Contrarily, all the descendants from parents without a TGR-1551 introgression for this region (BC_3 plants 28 and 37) were susceptible. Otherwise, BC_3 plant 198, whose BC_3S_1 offspring was susceptible to *Px*, was homozygous for the BO allele at the *locus cysdv63* and heterozygous at the position of the marker *CMPSNP464* (**Table 1**)*,* setting in this marker the lower limit of the candidate resistance region. Moreover, BC_3-198 also carried a TGR-1551 introgression at chromosome 12 that seemed to be unrelated to the recessive resistance (**Table 1**). On the other hand, the family BC_3S_1-146 was also susceptible but it segregated at the position of the marker *cysdv63,* whereas it was homozygous for the BO allele at the *locus cysdv65* (chr5: 26,408,895 bp), narrowing the candidate interval to a region of ~622 kb between markers *cysdv63* and *CMPSNP464.* This same region had been described as related to CYSDV resistance derived from 'TGR-1551´ (Pérez-de-Castro *et al.,* 2020).

To narrow the candidate interval in chromosome 5, the heterozygous plants for this region—BC_4-159 plants 4 and 11—were selected. The plant BC_4(159)4 was crossed with 'Piel de Sapo' (PS) to obtain the BC_4(159)4xPS offspring. It was also backcrossed to BO to obtain the BC_5(159)4 progeny. In the case of the plant BC_4(159)11, only the BC_5 offspring backcrossed to BO were obtained. These families (approximately six plants each) were genotyped with the HRM markers *cysdv63* and *cysdv65* and those plants that were heterozygous for both markers were selected and genotyped with background markers 2 set in the Agena Bioscience platform. The plants with the higher genetic background for the BO or PS genome were selected and self-pollinated. Families BC_4(159)4xPS-7S_1, BC_4 (159)4xPS-18S_1, BC_5(159)4-14S_1, and BC_5(159)11S_1 were phenotyped for the resistance to races 1, 2, and 5 of CPM (20 plants per family). They were also genotyped with the HRM markers *cysdv63* and *cysdv65.* The phenotyping/genotyping results of these offspring were compatible with the interval for the dominant gene obtained in the BC_3 and BC_3S_1 analyses. Moreover, three plants were found to be recombinant in the candidate region: (BC_4(159)4xPS-7S_1 plants 2 and 13 and BC_5(159)4-14S_1 plant 5). These plants were also genotyped with the HRM markers *cysdv626* (chr5:

26,058,307 bp) and *cysdv6531* (chr5: 26,309,060 bp) to narrow the interval (**Table 2**). These three plants were heterozygous at the position of the marker *cysdv626* and homozygous for the BO allele for the marker *cysdv65*. For the marker *cysdv6531*, BC_4(159)4xPS-$7S_1$ plant 2 was heterozygous, while plants BC_4(159)4xPS-$7S_1$-13 and BC_5(159)4-$14S_1$-5 were homozygous for the BO allele. While the BC_4(159)4xPS-$7S_1$ plants 2 and 13 were resistant to CPM, BC_5(159)4-$14S_1$ plant 5 was susceptible. These phenotyping/genotyping results enabled us to narrow the candidate interval to a region of 250,753 bp between markers *cysdv626* and *cysdv6531* (**Figure 1**). There are 34 predicted genes in the candidate region (**Supplementary Table S3**). GBS data were used to perform an SNP-calling analysis and SNPeff was used to predict the effects of genetic variants found between TGR-1551 and BO in this candidate region. SNPs were only found in two candidate genes, *MELO3C004297* and *MELO3C004311*, that coded a branched-chain-amino-acid aminotransferase-like protein and a TMV resistant protein N-like, respectively. The detected SNPs had a modifier-predicted impact on the coded protein functions (**Supplementary Table S4**). One of these candidate genes, *MELO3C004311*, has also been proposed as the gene conferring resistance in the accession MR-1 (Branham *et al.,* 2021).

Several BC_5S_1 plants, which were homozygous for the TGR-1551 alleles at the candidate region of chromosome 5, were self-pollinated to obtain their BC_5S_2 offspring. These BC_5S_2 families were phenotyped for resistance against *Px* races 1, 2, and 5 and all of them resulted in being resistant, validating the previously obtained results.

Table 2. Genotype for SNP markers in the candidate region of chromosome 5 of those BC_4xPS-S1 and BC_5S_1 plants that were recombinant between markers *cysdv63* and *cysdv65* (A: homozygous for 'Bola de Oro´ allele; H: heterozygous). The phenotype of the genotyped plants is indicated. The physical position of the SNPs in version v.4.0 of the melon genome (available at https://melonomics.net/ (accessed on 1ST June 2022)) is indicated.

Marker name	Chr[a]	Genomic position (bp) v.4.0	BC_4(159)4xPS-$7S_1$-13	BC_4(159)4xPS-$7S_1$-2	BC_5(159)4-$14S_1$-5
Cysdv63	5	25,782,654	H	H	H
Cysdv626	5	26,058,307	H	H	H
Cysdv6531	5	26,309,060	A	H	A
Cysdv65	5	26,408,895	A	A	A
Phenotype			Resistant	Resistant	Susceptible

[a]: chromosome

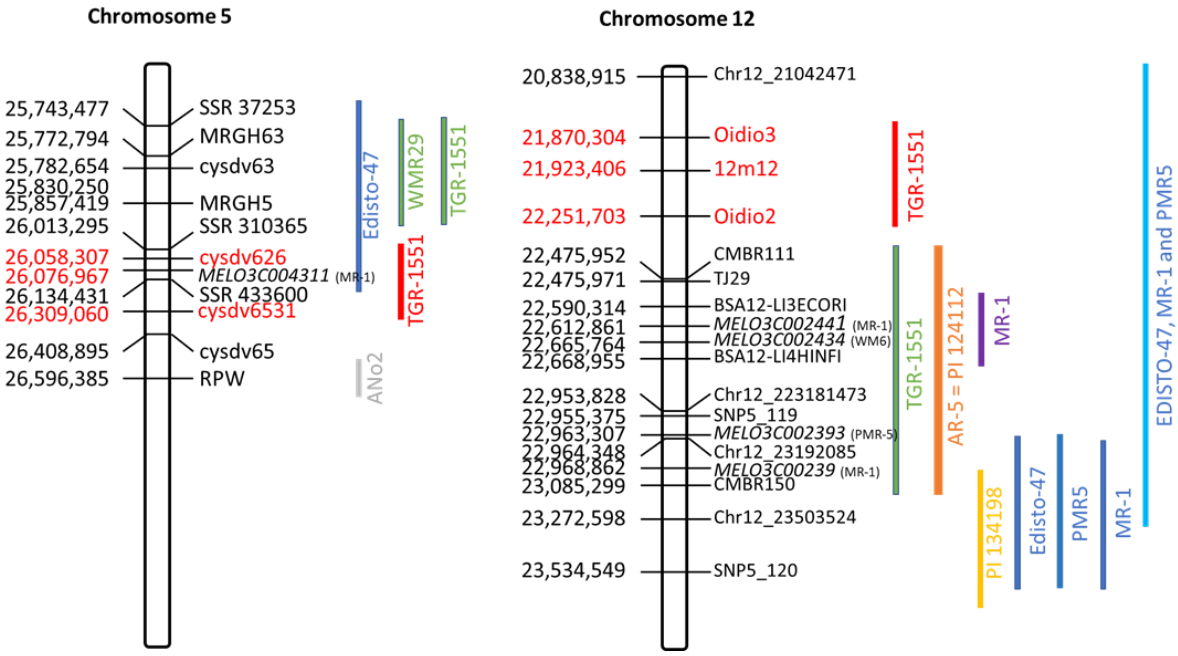

Figure 1. Genomic regions associated with the resistance to powdery mildew (*P. xanthii*) derived from different sources. The results obtained in this work are marked in red and the previous results derived from TGR-1551 are represented in green. The proposed candidate genes for other resistance sources are also indicated. The presented physical positions are referred to the genome version v.4.0 of *C. melo*.

2.3. Narrowing of the Candidate Region on Chromosome 12

Eight BC_3 plants that only had a TGR-1551 introgression in chromosome 12 (BC_3 plants 34, 78, 88, 96, and 139), or with an introgression not related to CPM resistance in chromosome 5 (BC_3 plants 24, 89, and 148), were analyzed. The selfing progenies of two of these plants heterozygous for markers *CMPSNP285* (chr12: 20,421,309 bp) and *CMPSNP361* (chr12: 23,000,406 bp) (BC_3 plants 34 and 139) segregated for the resistance in question, while the descendants of plant BC_3-88 were susceptible. Four recombinant plants were found according to these two markers (BC_3 plants 78, 89, 96, and 148) (**Table 1**). On the one hand, BC_3-78 was heterozygous for the marker *CMPSNP285* and homozygous for the BO allele for the marker *CMPSNP361* and its offspring segregated for resistance. On the other hand, the BC_3 plants 89, 96, and 148 were homozygous for the BO allele for the marker *CMPSNP285* and heterozygous for the marker *CMPSNP361* with susceptible, segregant, and susceptible progenies, respectively. The high level of recombination in this region demonstrated that the BC_3S_1-88 offspring were susceptible to CPM

even though the BC_3-88 plant was heterozygous for both markers in chromosome 12. These results allowed us to initially narrow the candidate interval to a 2.6 Mb region between markers *CMPSNP285* and *CMPSNP361*.

Remarkably, those BC_3S_1 families whose parents had the TGR-1551 introgressions at both candidate regions of chromosomes 5 and 12 (BC_3 plants 95, 105 and 141) had a higher ratio of resistant plants, which agrees with a dominant–recessive epistatic model.

Based on GBS data previously obtained by the research group, 10 high-resolution melting markers (HRM) were designed to narrow the candidate region in chromosome 12 (**Supplementary Table S5**). These HRM markers were evenly distributed throughout the interval between markers *CMPSNP285* and *CMPSNP361*. Six BC_3 plants and their BC_3S_1 offspring, which had previously been phenotyped for resistance to CPM (BC_3S_1 families 34, 88, 89, 96, 139, and 148), were genotyped with these 10 HRM markers (Table 3). These results showed that a recombination event had taken place within markers *CMPSNP285* and *CMPSNP361* in the BC_3-88 plant. Only those BC_3S_1 families whose parents were heterozygous for both markers *Oidio2* (chr12: 22,251,703 bp) and *Oidio1* (chr12: 22,309,972 bp) (BC_3S_1 families 34, 96, and 139) segregated for the resistance to CPM. The plant BC_3-139 was homozygous for the BO allele at the position of the marker *Oidio3* (chr12: 21,870,304 bp) and its selfing progeny was segregant for resistance, setting the upper limit of the candidate region at the position of the *locus Oidio3*. BC_3 plants 88 and 148 were homozygous for the BO allele for the markers *Oidio3*, *Oidio2,* and *Oidio1* and heterozygous for the marker *OidioC* (chr12: 22,875,711 bp). Their offspring were susceptible to CPM, narrowing the candidate interval to a ~1 Mb region between markers *Oidio3* and *OidioC*.

The genotyping of the $BC3S_1$ families that segregated for CPM resistance showed significative phenotypic differences between those plants that were homozygous for the TGR-1551 allele for the markers *Oidio2* and *Oidio1* and those that were heterozygous or homozygous for the BO allele at these positions.

Afterwards, BC_4-95 plant 10 was selected, as it was heterozygous at the candidate region of chromosome 5 and at the position of the marker *SNP361*. It was self-pollinated and crossed with the PS cultivar BGCM-126 to obtain the $BC_4(95)10S_1$ and $BC_4(95)10$xPS offspring, respectively. Six plants of the $BC_4(95)10$xPS offspring were genotyped with a new set of 160 SNP markers evenly distributed throughout the genome (Background marker 2) (**Supplementary Table S1**). Moreover, these plants were also genotyped with the HRM markers *cysdv63* and *cysdv65* (**Table 3**).

Those plants homozygous for the BO allele at the candidate region of chromosome 5 and heterozygous for the marker *CMPSNP361* at chromosome 12 were selected (BC_4(95)-10xPS plants 3 and 5). These two plants were also genotyped with the HRM markers *Oidio3, Oidio2,* and *Oidio1* (**Table 4**). Both were homozygous for the BO allele at the position of the markers *Oidio3* and *S12_22130778* (background marker 2, chr12: 21,923,406 bp) and heterozygous for markers *Oidio2* and *Oidio1.* Their selfing progenies were evaluated for their resistance to races 1, 2, and 5 of CPM. All the BC_4(95)-10xPS-S_1 plants were susceptible, independently of their genotype for the *Oidio2* marker. This allowed us to narrow the candidate interval to a 381,399 bp region between markers *Oidio3* and *Oidio2* (Figure 1). This region had 50 annotated genes, some of which could be good resistance candidates (**Supplementary Table S5**).

Table 3. SNP markers used to narrow the candidate interval of chromosome 12 (A: homozygous for the 'Bola de Oro' allele; H: heterozygous). The molecular markers in the candidate regions are in bold format. The position of the SNPs according to the last version of the genome (v.4.0, available at http//www.melonomics.net accessed on 1 June 2022) is indicated. The phenotype of the genotyped plants is indicated (SU: susceptible).

			BC_4(95)10xPS Plants	
Marker	**Chr[a]**	**Genomic position v.4.0(bp)**	**3**	**5**
S5_23380459	5	24,192,281	A	A
cysdv63	5	**25,782,654**	**A**	**A**
cysdv65	5	**26,408,896**	**A**	**A**
S5_25653869	5	26,419,648	A	A
S12_18721450	12	18,530,394	A	A
Oidio3	**12**	**21,870,304**	**A**	**A**
S12_22130778	**12**	**21,923,406**	**A**	**A**
Oidio 2	**12**	**22,251,703**	**H**	**H**
Oidio1	12	22,309,972	H	H
CMPSNP361	12	23,000,406	H	H
Phenotype			SU	SU

[a]: chromosome

SNPeff was used to annotate and predict the effects of the SNPs found in two GBS assays between TGR-1551 and BO within this region. SNPs were detected in the sequence of 19 candidate genes in this region. Most of those SNPs had a predicted modifier impact (**Supplementary Table S4**). Ten of the affected genes coded a serine/threonine-protein kinase. SNPs were also detected in genes coding (3S,6E)-

nerolidol synthase 1-like (*MELO3C002520*), a eukaryotic translation initiation factor-like protein (*MELO3C002515*), a BTB/POZ domain-containing protein At3g22104 (*MELO3C002514*), a protein detoxification (*MELO3C002526*), purine permease 3-like (*MELO3C002545*), an unknown protein (*MELO3C035727*), and three receptor-like protein kinases (*MELO3C002504, MELO3C002541,* and *MELO3C002538*) (**Table 3**).

Table 4. Genotype for SNP markers in the candidate region of chromosome 12 for the BC3 plants selected for not having a TGR-1551 introgression at chromosome 5 (A: homozygous for 'Bola de Oro' allele; H: heterozygous). The phenotype of the $BC3S_1$ progenies is indicated (SU: susceptible; SE: segregating). Markers in the candidate interval are in bold format.

			Number of BC_3 Plant					
Marker	**Chr [a]**	**Genomic Position (bp)**	**34**	**96**	**139**	**88**	**89**	**148**
CMPSNP285	12	20,421,309	H	A	H	H	A	A
Oidio8	12	20,920,054	H	A	A	n.d [1]	A	A
Oidio5	12	21,470,961	H	H	A	n.d	A	A
Oidio4	12	21,866,445	H	H	A	n.d	A	A
Oidio3	**12**	**21,870,304**	**H**	**H**	**A**	**A**	**A**	**A**
Oidio2	**12**	**22,251,703**	**H**	**H**	**H**	**A**	**A**	**A**
Oidio1	**12**	**22,309,972**	**H**	**H**	**H**	**A**	**A**	**A**
OidioC	**12**	**22,875,711**	**H**	**H**	**H**	**H**	**A**	**H**
OidoB	12	22,913,643	H	H	H	H	H	H
OidioA	12	22,915,510	H	H	H	H	H	H
CMPSNP361	12	23,000,406	H	H	H	H	H	H
Phenotype			SE	SE	SE	SU	SU	SU

[a]: chromosome. [1]: missing data

2.4. Changes in Resistance Levels Due to Low Temperatures

Fifty-seven $BC_4(95)10S_1$ plants and five PS plants were phenotyped for the resistance against race 5 of *Px*. Twelve days after inoculation, the plants of the PS line showed severe infection symptoms. Different levels of fungal sporulation were recorded for the $BC_4(95)10S_1$ plants. Thus, the segregation ratio of the $BC_4(95)10S_1$ plants observed was 34 resistant:23 susceptible, which is not consistent with a 13:3 ratio corresponding to the independent segregation of two genes, one dominant gene, and one recessive gene. These plants were genotyped with the HRM markers *cysdv63, cysdv65, Oidio3, Oidio2,* and *Oidio1.* All the plants were homozygous for the BO allele at the position of the marker *Oidio3*, while segregation was observed

for the rest of them. The combined effect of the QTLs in chromosomes 5 and 12 was analyzed (**Figure 2**). Those plants that were homozygous for the BO allele at the candidate region in chromosome 5 were only resistant when they were homozygous for the TGR-1551 allele at the *locus Oidio2* (**Figure 2A**). Those plants that were homozygous for the TGR-1551 alleles in the candidate region of chromosome 5 showed mild or no symptoms independently of their genotype at the position of the marker *Oidio2* (**Figure 2B**). There were 25 heterozygous plants at the position of the markers *cysdv63* and *cysdv65* that were heterozygous or homozygous for the BO allele at the position of the marker *Oidio2* in chromosome 12. Among those plants, there were 10 that showed a susceptible phenotype (**Figure 2C**). The resistance response of the heterozygous plants might have been affected by the lower temperatures recorded during the assay (the medium temperature was lower than 21 °C).

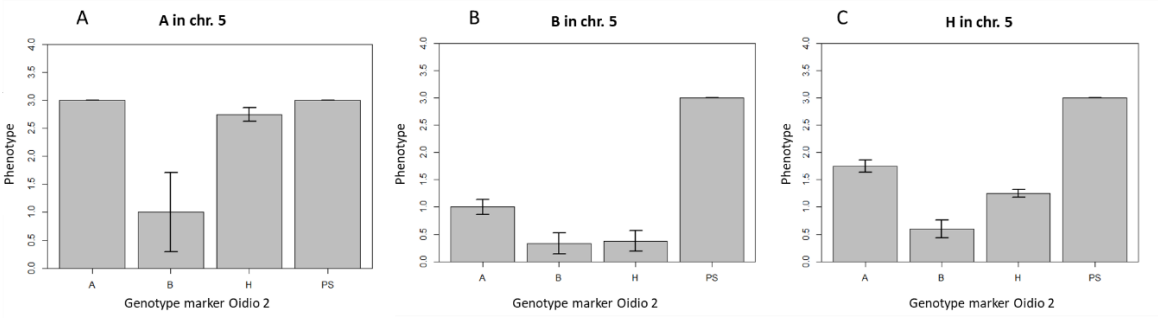

Figure 2. Interaction between QTL on chromosome 5 (markers *cysdv63* and *cysdv65*) and QTL on chromosome 12 (marker *Oidio2*) for symptom score at 12 days post-inoculation (bars indicate standard error). Genotype for marker *Oidio2*: BO: homozygous for 'Bola de Oro' allele; H: heterozygous: TGR: homozygous for TGR-1551 allele. Genotype for marker *cysdv63*: A: homozygous for 'Bola de Oro' allele (**A**); B: homozygous for TGR-1551 allele (**B**); H: heterozygous; PS: Piel de Sapo control (**C**).

3. Discussion

In this work, advanced backcross lines derived from an initial cross between TGR-1551 and 'Bola de Oro' were used to narrow the candidate regions of the resistance genes to *Px* and to develop PCR-based markers tightly linked to this resistance. Moreover, thanks to the genotyping achieved via the sequencing of both parents, candidate resistance genes have been provided.

Resistance to *Px* races 1, 2, and 5 derived from TGR-1551 was initially described as independently controlled by one dominant gene on chromosome 5 and a recessive one (Yuste-Lisbona *et al,* 2010, 2011). The phenotyping of an F_2 population derived

from the same cross and its genotyping through Bulked Segregant Analysis (BSA) and AFLP markers allowed for the identification of two QTLs, namely, *Pm-R1-2* and *Pm-R5*, the first of them related to resistance to races 1 and 2 and the latter to resistance against race 5 (Yuste-Lisbona *et al.,* 2011). Two resistance-like genes, *MRGH5* and *MRGH63*, belonging to the NBS-LRR (Nucleotide-binding site and leucine-rich repeats) family, were identified as candidate genes for CPM resistance in the TGR-1551 in the region of chromosome 5. Moreover, the molecular markers developed by Yuste-Lisbona *et al*. (2011) were also linked to the resistance derived from WMR29. The use of advanced backcrossed lines and the genotypic information derived from New Generation-Sequencing technologies (NGS) allowed us to narrow the candidate region related to the resistance to the three races to a 251 kb region between the HRM markers *cysdv626* (chr5: 26,058,307 bp) and *cysdv6531* (chr5: 26,309,060 bp). This narrowed candidate region contains 34 predicted genes, of which 31 were annotated (**Supplementary Table S3**), but does not include *MRGH63* and *MRGH5*, which are approximately <275 kb from the candidate interval (**Figure 1**). Several QTLs linked to *Px* resistance have been mapped in chromosome 5 (*qPx1-5, Pm-AN, Pm-w, Pm-Edisto47-2,* and *PmV-1-Pi124112*) (Branham *et al.*, 2021; Ning *et al.*, 2014; Perchepied *et al.*, 2005; Périn *et al.*, 2002b; Pitrat, 1991; Wang *et al.*, 2011) but only the QTLs *qPx1-5* and *Pm-AN*, linked to resistance to *Px* race 1 derived from MR-1 and Ano2, respectively, overlapped with the proposed candidate region (Branham *et al.*, 2021; Wang *et al.*, 2011) (**Figure 1**). Moreover, the region in which these resistances are located overlaps with a 760 kb region with the highest concentration of resistance genes in the melon genome (González *et al.*, 2013). Furthermore, this region has been proven to be highly variable both at the intra- and interspecific levels, explaining the differences in resistance found in different melon genotypes (González *et al.*, 2013).

Most of the annotated genes within the candidate region have resistance-related functions. Among them, there are two genes annotated as `disease resistance proteins´ (*MELO3C004320* and *MELO3C031556*), as well as genes related to host pattern recognition receptors (PRR) such as two NBS-LRRs (*MELO3C004319* and *MELO3C031325*) and a `receptor-like cytosolic serine/threonine-protein kinase RBK2´ (*MELO3C004315*). The TMV resistance protein N-like, a TIR-NBS-LRR gene possessing homology with resistance genes (Marathe *et al.*, 2002), was frequently annotated (seven of the annotated genes in the candidate interval). As the dominant resistances of powdery mildew identified so far have been easily broken by newly emerging races (Cui *et al.*, 2022b), it has been postulated that many of the QTLs detected could encode PRRs that would be manipulated by the secreted

fungal effectors (Pedersen *et al.*, 2012; Toruño *et al.*, 2016). It has recently been proposed that several dominant resistances against powdery mildew in melon could be caused by missense mutations in different LRR-receptor like kinases and TIR-NBS-LRR genes (Branham *et al,* 2021; Hong *et al.,* 2022). In this sense, two SNPs with a predicted modifier impact were detected within the sequence of a gene coding TMV resistance protein N-like (*MELO3C004311*) (**Supplementary Table S4**), which could play a potential role in an eventual protein product and thereby confer resistance against*Px*. This TIR-NBS-LRR gene has also been proposed as the candidate gene, derived from MR-1, conferring resistance against *Px* race 1 (Branham *et al.,* 2021). Moreover, six variants with modifier impacts were found within the sequence of the predicted gene *MELO3C004297*, which was annotated as a `Branched-chain-amino-acid aminotransferase-like protein´ (**Supplementary Table S4**). These kinds of proteins are related to defense responses in several species. A branched chain amino acid aminotransferase, termed TaBCAT1, has been described as a positive regulator of wheat rust (*Puccinia triticina)* susceptibility, as it had a key role in SA-dependent defense activation (Corredor-Moreno *et al.*, 2021). In *A. thaliana*, the levels of the branched-chain amino acid-related compound ILA correlate with the expression of a glucosyltransferase (UGT76B1) that is induced in response to stress (von Saint Paul *et al.*, 2011). Therefore, these two genes are the principal candidate resistance genes in the proposed interval.

In addition to the previously cited R genes, two predicted genes were noted to have functions related to cell wall formation (*MELO3C002509* and *MELO3C002512*). The cell wall is one of the primary shields against pathogen infections. It acts as a barrier that pathogens must degrade for infection progression, and it also contains antimicrobial compounds (Miedes *et al.*, 2014; Bacete *et al.*, 2018). Moreover, cell wall compounds that are released during infection can act as damage-associated molecular patterns (DAMPs), triggering plant immune responses thanks to their perception by PRRs (de Azevedo Souza *et al.*, 2017; Voxeur *et al.*, 2019). It has been proven that a loss of susceptibility factors increases cell wall thickness, leading to a more effective defense response against powdery mildew (Hong *et al.*, 2015; Nie *et al.*, 2015; Vogel *et al.*, 2002, 2004). Moreover, the resistance derived from TGR-1551 to *Px* could be related to post-inoculation modifications of the cell walls, since, when comparing the response of TGR-1551 and BO against infection, a 50% increase in the number of cells with an accumulation of callose in their cell walls is observed in TGR-1551 (Beraldo-Hoischen *et al.*, 2021). There were also two predicted genes related to photosynthetic processes (*MELO3C004308* and *MELO3C004307*). During powdery mildew infection, the genes involved in photosynthesis and related processes are upregulated in melons (Polonio *et al.*,

2019). The candidate region also includes the virus aphid transmission resistance gen (*Vat*) (ten of the predicted genes are annotated as `Vat protein´) (González *et al.,* 2013) carried by TGR-1551 (Sarria-Villada *et al.*, 2009). Moreover, the resistance to CYSDV derived from TGR-1551 has been mapped in the same region (Pérez-de-Castro *et al., 2020*), which would explain why—while developing a resistant line to CYSDV—the selected plants resulted in being resistant to *Px* and *Aphis gosypii* as well, even though no previous selection against the fungus nor the aphid had been made (Palomares-Rius *et al.*, 2018).

Regarding the recessive gene, in a previous work, an RIL population derived from a cross between TGR-1551 and BO was evaluated for resistance to the three races of *Px* and a QTL associated with the recessive gene that was found in chromosome 12 (Beraldo-Hoischen *et al.*, 2012). The SSR markers CMBR111 (chr12: 22,475,952–22,476,042 bp), TJ29 (chr12: 22,475,971–22,476,086 bp), and CMBR150 (chr12: 23,085,299–23,085,068 bp) were found to be linked to this QTL (Beraldo-Hoischen *et al.*, 2012). The same SSR markers were also linked to the resistance QTL(AR5) derived from AR5 (Fukino *et al.,* 2008). Nevertheless, the phenotyping and genotyping of the advanced backcrossed lines allowed us to narrow the resistance interval to a region of approximately 400 kb between HRM markers *Oidio3* (chr12: 21,870,304 bp) and *Oidio2* (chr12: 22,251,703 bp) (**Table 3**). This narrowed region is not located within the previously proposed resistance QTL and is also outside the boundaries proposed for other resistance sources such as Edisto27, MR-1, PMR5, WM6, PI 124112, and PI 134198 (Branham *et al.*, 2021; Cao *et al.*, 2021; Cui *et al.*, 2022a; Fukino *et al.*, 2008; Hong *et al.*, 2022; Howlader *et al.*, 2020; Li *et al.*, 2017; Liu *et al.*, 2010a; Natarajan *et al.*, 2016) (**Figure 1**). Nevertheless, the recessive gene present in TGR-1551 is unique, since no recessive genes conferring resistance to more than one *Px* race have been reported previously in melons, and this resistance could be conferred by a gene different from those derived from other accessions.

The candidate interval contains 50 predicted genes and all of them have been annotated (**Supplementary Table S6**). Most of the annotated genes have defense-related functions, which might indicate the presence of a resistance cluster. `Kinase binding´ is the molecular function most frequently described, and several predicted genes were also annotated as `cysteine-rich receptor-like protein kinase´. There are two predicted genes annotated as `transcription factors´ (*MELO3C002551* and *MELO3C002522*) and another as a `eukaryotic translation initiation factor-like protein´ (*MELO3C002515*). Moreover, *MELO3C002553* was annotated as a `sugar carrier protein C-like´. Sugar metabolism plays a key role in the plant–pathogen interaction, as bacteria and fungus tend to acquire these metabolites during

infection. Modifications in sugar metabolism during infection plays a key role in the susceptibility to all wheat rust and powdery mildew pathogen species in wheat (Moore *et al.*, 2015). In grapevine, Hexose Transporter 5 (VvHT5) is strongly upregulated in coordination with Cell Wall Invertase (VcwINV) during powdery and downy mildew infections (Hayes *et al.,* 2010). The predicted gene *MELO3C002550* has been annotated as a `Flowering locus T/terminal flower 1-like protein´. Common genetic and epigenetic regulators for flowering and systemic acquired resistance (SAR) have recently been suggested (Banday & Nandi, 2015). There is also one predicted gene implicated in photosynthetic processes (*MELO3C002510*).

The effect of the detected SNPs was calculated with SNPeff (**Supplementary Table S4**). Regarding other accessions, different candidate resistance genes have recently been proposed using a similar approach (Branham *et al.*, 2021; Cao *et al.*, 2021; Cui *et al.*, 2022ª; Hong *et al.*, 2022). There were SNPs affecting the coding region of 19 genes located within the candidate region on chromosome 12. Most of those SNPs were located in sequences of genes coding serine/threonine-protein kinases (STKs) and receptor-like serine/threonine-protein kinases (RLKs) (**Supplementary Table S4**) and frequently had a predicted modifier impact over the protein function. RLKs are key components of the plant immune system, acting in both broad-spectrum, elicitor-initiated defense responses and as dominant resistance genes in race-specific pathogen defense (Ramonell & Goff, 2007). Moreover, STK domains not only interact with avirulence proteins but also function as signal transduction mediators (Martin *et al.*, 1993; Song *et al.*, 1995). Mutations in these proteins could lead to a better recognition of PAMPs or DAMPs and, hence, to a faster plant response against *Px*. A similar phenomenon occurs with serine/threonine-protein kinases, which play a key role in the transduction of the internal and external signals. Some genes coding these kinds of receptors, and located in chromosome 12, have already been proposed as candidate resistance genes derived from MR-1 and PMR5 (Branham *et al.*, 2021; Cui *et al.*, 2022a; Hong *et al.*, 2022). Nevertheless, other candidate genes have been proposed within the same region (Cao *et al.*, 2021; Cui *et al.*, 2022a). In addition to the mutations previously described, an SNP was also detected within the coding region of a purine permease. These proteins are involved in cytokinin transport and it has been proven that the suppression of CK transporters can lead to the suppression of the immune response (Wang *et al.*, 2017). There was also an SNP that caused the appearance of a new stop codon in the sequence of a gene coding a BTB/POZ domain-containing protein, At3g22104. These proteins are involved in the pathway of protein ubiquitination, and the reduction in ubiquitin levels enhanced the susceptibility to powdery mildew in barley (*Hordeum vulgare)* (Dong *et al.*, 2006). Moreover, other components of the

ubiquitination complex have also been related to the plant defense against fungi (Cui *et al.*, 2022a; Li *et al.*, 2011). An SNP with a predicted modifier impact was also found within the 3'UTR region of a gene coding a eukaryotic translation initiation factor (EIF). These proteins have been widely described as susceptibility factors in viral infections, but not similar functions have been documented in plant–fungal interactions. Finally, an SNP that caused the loss of a start codon was also detected within the sequence of a gene coding a (3S,6E)-nerolidol synthase 1-like protein. These proteins are involved in monoterpene and sesquiterpene biosynthesis. The enhanced production of some sesquiterpenes has been related to the inhibition of bacterial growth (Huang *et al.*, 2012), and many monoterpene-induced transcripts are annotated as either transcription factors or defense genes (Godard *et al.*, 2008).

A comparative transcriptome profiling of genes and pathways related to resistance against powdery mildew was performed in the contrasting melon genotypes MR-1 and Topmark (Zhu *et al.*, 2018). The differentially expressed genes (DEGs) detected were classified into seven functional groups: pathogen recognition, signal transduction, transcription factors (TFs), phytoalexin biosynthesis, other primary metabolite functions, Mildew Locus O genes (MLOs), and pathogenesis-related (PR) proteins. The defense-related genes showed an increased expression at the early stage of *Px* infection in the resistant genotype MR-1, whereas the defense response was suppressed in the susceptible cultivar. Moreover, the expression changes tended to be maintained during the infection (Zhu *et al.*, 2018). There are also several non-coding RNAs (long non-coding RNAs (lncRNAs) and circular RNAs (circRNAs)) (Sun *et al.*, 2021; Zhou *et al.*, 2020) that have been described as being related to *Px* resistance, promoting the expression of genes involved in defense processes. Therefore, the resistance to *Px* in melons comprises many defense-related mechanisms. The regions presented herein as linked to the resistance derived from TGR-1551 contain several genes involved in the detection of fungal elicitors, signal transduction, and resistance response. A transcriptomic analysis would be needed to identify differentially expressed genes in the candidate regions and further VIGs assays will be performed to functionally validate the proposed candidate genes.

Resistance to powdery mildew has been described as temperature-dependent in different species, including melons and cucumbers (Beraldo-Hoischen *et al.*, 2021; Fukino *et al.*, 2013; Hosoya *et al.*, 2000; Tores *et al.*, 1996). The phenotyping/genotyping results of the $BC_4(95)10S_1$ offspring revealed that the resistance conferred by the QTL located in chromosome 5 may decrease under low temperature conditions in heterozygous lines. The plants homozygous for the TGR-

1551 allele at *locus Oidio2* were not affected by low temperatures, whereas those plants that were heterozygous for the markers *cysdv63* and *cysdv65* and heterozygous or homozygous for the BO allele at *locus Oidio2* showed a decrease in their resistance response (**Figure 2C**). This condition had not previously been observed in the assays carried out in the same season of different years with higher temperatures. These results were consistent with those observed by Beraldo-Hoischen *et al*. (2021), where TGR-1551 and the NIL21, carrying the QTL Pm-R derived from TGR-1551, were susceptible when inoculated at low temperatures. Under low temperature conditions, a delay in the resistance response was observed and the number of cells with callose accumulation per point of penetration decreased. A similar behavior was observed in the resistant cucumber line PI 1970088-1, where a major QTL was always detected under high and low temperature conditions and four other QTLs were temperature-dependent (Sakata *et al.*, 2006). Thus, the resistance conferred by the dominant QTL located in chromosome 5 would be temperature-dependent only for the heterozygous plants, while the temperature would not affect the resistance provided by the recessive QTL located in chromosome 12.

4. Materials and Methods

4.1. Plant Material

The resistance source used in this study, TGR-1551, is a Zimbabwean melon cultivar described as resistant to *Px* races 1, 2, and 5. Generations used in this study come from the breeding program aimed at the introgression of the resistance derived from TGR-1551 to commercial types via backcrosses to the Spanish melon cultivars 'Bola de Oro' and 'Piel de Sapo'. The populations inoculated were constructed from an original BC_3 population composed of 200 plants obtained from the F_1 TGR-1551 x 'Bola de Oro' and three successive backcrosses to the susceptible cultivar 'Bola de Oro' (BO). BC_3 plants were selected for carrying the candidate regions previously described as linked to *Px* resistance and with less genetic background from TGR-1551. New backcrosses to BO and self-pollinations were carried out in consecutive years (**Supplementary Figure S1**). Initially, 200 BC_3 plants were genotyped with markers linked to the candidate resistance region to *Px* at chromosome 5 (see details below) in 2018. Twenty BC_3 plants were selected, backcrossed to BO, and self-pollinated to obtain their corresponding BC_4 and BC_3S_1. In 2019, the BC_3S_1 and BC_4 families generated were genotyped to confirm the presence of the genomic regions associated with resistance to CPM in chromosome 5 and 12. The 20 selected BC_3S_1 were artificially inoculated with *P. xanthii*. The corresponding BC_4 were then backcrossed to BO and to the CPM-susceptible Spanish cultivar 'Piel de

Sapo' (PS) to obtain BC_5 and BC_4 x PS, respectively. In 2020, plants of those populations were genotyped with background and HRM markers to confirm the presence of the genomic regions of interest. Three BC_5 and four BC_4 x PS plants were selected and self-pollinated to obtain BC_5S_1 and BC_4 x PS-S_1 families, respectively. In 2021, the BC_5S_1 and BC_4 x PS-S_1 families were genotyped with HRM markers and phenotyped for CPM resistance to narrow the genomic locations of the resistance to *Px* (**Table 1**). BC_5S_1-resistant plants that were homozygous for the TGR-1551 allele at the candidate region of chromosome 5 were self-pollinated and their BC_5S_2 progenies were phenotyped for CPM resistance.

4.2. Artificial Inoculations

The isolates of *Podosphaera xanthii* used in this experiment were SF30 (race 1), P-15.0 (race 2 F), and C8 (race 5). They were all collected in melon crops growing under greenhouses in the South of Spain. For each case, and with the help of a brush, a small amount of conidia was taken from the natural mycelial surface present on the melon leaves and placed on cut and previously disinfected cotyledons of cucumber var. 'Black Beauty' growing on water agar medium (0,8% agar, 3% benzimidazole, and 3.2% sucrose) in petri dishes under axenic conditions, as described by Yuste-Lisbona *et al*. (2011a). Cotyledons were disinfected (solution of NaClO at 20gr/L) before use to allow a prolonged preservation of both cotyledon and mildew, avoiding possible contamination of the powdery mildew colonies. Once the pathogen was identified as *P. xanthii* based on its morphological characters under light microscope examination, monosporic cultures were performed. Natural powdery mildew is composed of a mixture of different genotypes whose competitivity, in particular under in vitro culture conditions, can vary. So, cloning by single conidia isolation seemed to be necessary. An agar-coated eyelash, moistened by medium contact, permitted the straightforward capture of individual conidia under binocular lens, which were then deposited on cotyledons maintained under axenic conditions in individual petri dishes. Colonies appeared after about 15 days under incubation conditions in growth room with controlled temperature (23 °C) and relative humidity (38–65%) at a photoperiod of 16/8 h (light/dark). The races of the different fungal isolates were confirmed by analyzing the reactions of a set of differential melon lines to each isolate, as described by Bardin *et al*. (1999). Those colonies were then used to carry out the inoculations. The fungus was sub-cultured several times until the inoculation date. The monosporic cultures were maintained under the same conditions of incubation until the inoculation dates.

Three to five plants each from the resistant parental line TGR-1551, the susceptible parental line 'Bola de Oro', and their F_1 progeny, as well as a variable number of plants of the different generations (**Suplementary Figure S1**), were inoculated with races 1, 2, and 5 of *P. xanthii* on the same leaf. Due to availability of inoculum source and considering that response to the three races in TGR-1551-derived plant materials is homogeneous, two of the BC_4S_1 families of interest were inoculated solely with race 5 in 2020.

All plants were inoculated artificially by depositing a small amount of conidia from each race at two spots (at each side of, and equidistant from, the main leaf vein) on the second true leaf of each plant (Ferriere & Molot, 1988). Inoculation with the three races was carried out on the same leaf of each plant (**Supplementary Figure S2**).

The plants were maintained and inoculated in an insect-proof glasshouse during spring, from the middle of March to the middle of June, at temperatures ranging from 18 to 32 °C, the relative humidity ranging between 43–79%, and photoperiod ranging from 12:12 to 16:8 h (light:dark). That period of time is the most adequate to evaluate PM because it is the season when melons are grown under greenhouses in Spain as well as along the Mediterranean basin. Twelve days after inoculation, plants were scored according to the level of sporulation of the fungus, using a scale from 0–3, similar to those previously used by other authors for powdery mildew in melons (Bohn & Whitaker, 1964; Boiteux *et al.*, 1995; McCreight, 2003; Yuste-Lisbona *et al.*, 2011a,b) (**Supplementary Figure S2**). The following classes were established: class 0, no conidia germination; class 1, some conidia germination, no visible sporulation, and no disease progression; class 1.5, low level of sporulation, and the disease does not seem to progress; class 2, moderate level of sporulation and sparse mycelia but the disease progresses; class 2.5, clear sporulation; and class 3, profuse sporulation. Plants in classes 0 and 1 were considered resistant (R), those in class 1.5 were considered moderately resistant (MR), and those in classes from 2 to 3, where the infection progressed, were considered susceptible (S).

4.3. Molecular Markers and Genotyping Methods

Total DNA was extracted from young leaves following the method described by Doyle & Doyle, (1990) with minor modifications (Esteras *et al.,* 2013). DNA was quantified using spectrophotometry via a Nanodrop ND-1000 Spectrophotometer v.3.5 (LabTech International, Heathfield, UK) and adjusted to the concentrations suited for the different genotyping analyses.

Previously existing SNPs and new ones developed in this work were used to genotype the different segregating populations. Initially, 200 BC_3 plants were genotyped with three existing panels of SNP markers (CYSDV1, WMV1 and WMV2) implemented in the Agena Bioscience iPLEX®® Gold MassARRAY platform by the Epigenetic and Genotyping unit of the University of Valencia (Unitat Central d'Investigació en Medicina (UCIM), Valencia, Spain). These panels covered the candidate region on chromosome 5 and had been developed in previous studies (Pérez-de-Castro et al, 2019, 2020). 52 BC_3 plants were selected for carrying different introgression in the resistance region. The selected plants were then genotyped with an existing panel of 124 SNP markers evenly distributed throughout the genome (Background markers 1) (**Supplementary Table S1**), implemented in the Agena Bioscience iPLEX®® Gold MassARRAY platform. This SNP set had previously been validated in populations derived from ibericus x acidulus melon crosses (Esteras *et al.*, 2013; Leida *et al.*, 2015; Perpiñá *et al.*, 2016; Sáez *et al.*, 2017; Pérez-de-Castro *et al.*, 2019). 20 BC_3 plants which minimized the amount of donor parent genome in the background genome were selected. To increase the resolution of the candidate region of chromosome 5, the BC_3S_1 and BC_4 progenies of the 20 selected BC_3 plants were also genotyped with the panel sets CYSDV1 and CYSDV2 (Pérez-de-Castro *et al.,* 2020) (**Supplementary Table S2**). The lectures of a GBS experiment, conducted to perform genetic diversity studies (including TGR-1551 and BO, among many other genotypes), were mapped to the reference melon genome (v.4.0) using bowtie2 v.2.3.4 (Langmead & Salzberg, 2012) and an SNP calling was carried out with Freebayes v.1.3.4 (Garrison & Marth, 2012). A new set of 160 markers evenly distributed throughout the genome that allowed us to distinguish between the different melon groups was selected (**Supplementary Table S1**). These 160 markers provided complementary information to that offered by the previously used set of 124 markers. This new set was also implemented in the Agena Bioscience Platform and used to genotype the BC_4 x PS, BC_4S_1 and BC_5 progenies. To further narrow the candidate interval in chromosome 12, the marker *CMPSNP361* (**Supplementary Table S1**) and 10 new SNPs, retrieved from the GBS experiment and evenly distributed within the candidate interval, were adapted to a PCR-based protocol for their analysis by high-resolution melting (HRM) (**Supplementary Table S5**). For a better analysis of the melting curves provided by HRM markers, we avoided selecting SNPs that produce an A/T nucleotide change. These HRM markers were used to genotype selected BC_3S_1 and BC_4 x PS-S_1 families. The previously designed HRM markers *csydv63* and *cysdv65* (Pérez-de-Castro *et al.,* 2020)] were used to genotype the BC_3S_1, BC_4, BC_4 x PS, BC_4 x PS-S_1, BC_5, and BC_5S_1 selected families. To narrow the candidate region in chromosome 5, two additional

SNPs were adapted for analysis by HRM (*csydv626* and *cysdv6351)* and they were used to genotype the BC_4 x PS-S_1 and BC_5S_1 populations. The predicted effect of the SNPs' variants found between TGR-1551 and BO within the candidate regions was analyzed with SNPeff version 1.3.4 (Cingolani *et al.*, 2012).

5. Conclusions

Breeding new cultivars resistant to powdery mildew is the most efficient, durable, and environmentally respectful way to fight this pathogen. The African accession TGR-1551 has been reported as being resistant to CPM races 1, 2, and 5. This work has allowed for the narrowing of the candidate intervals of the dominant and recessive QTLs associated with the resistance to CPM derived from TGR-1551 to a region of approximately 250 kb and 381 kb, respectively. The SNP markers provided herein are a useful resource for the introgression of CPM resistance in commercial melon cultivars and several candidate genes have been proposed. Resistance to CPM is strongly affected by the environmental temperature. Thus, the availability of markers tightly linked to the resistance QTLs is essential for accelerating the introgression of CPM resistance into elite cultivars or landraces.

DECLARATIONS

Author Contributions: Conceptualization, M.L.G.-G., A.P.-d.-C. and B.P.; methodology, M.L.G.-G. and M.L.-M.; software, M.L.-M.; investigation, M.L.G.-G., B.P., M.L.-M. and A.P.-d.-C.; writing—original draft preparation, review and editing, M.L.-M., A.P.-d.-C., M.L.G.-G. and B.P.; funding acquisition, M.L.G.-G., A.P.-d.-C. and B.P. All authors have read and agreed to the published version of the manuscript.

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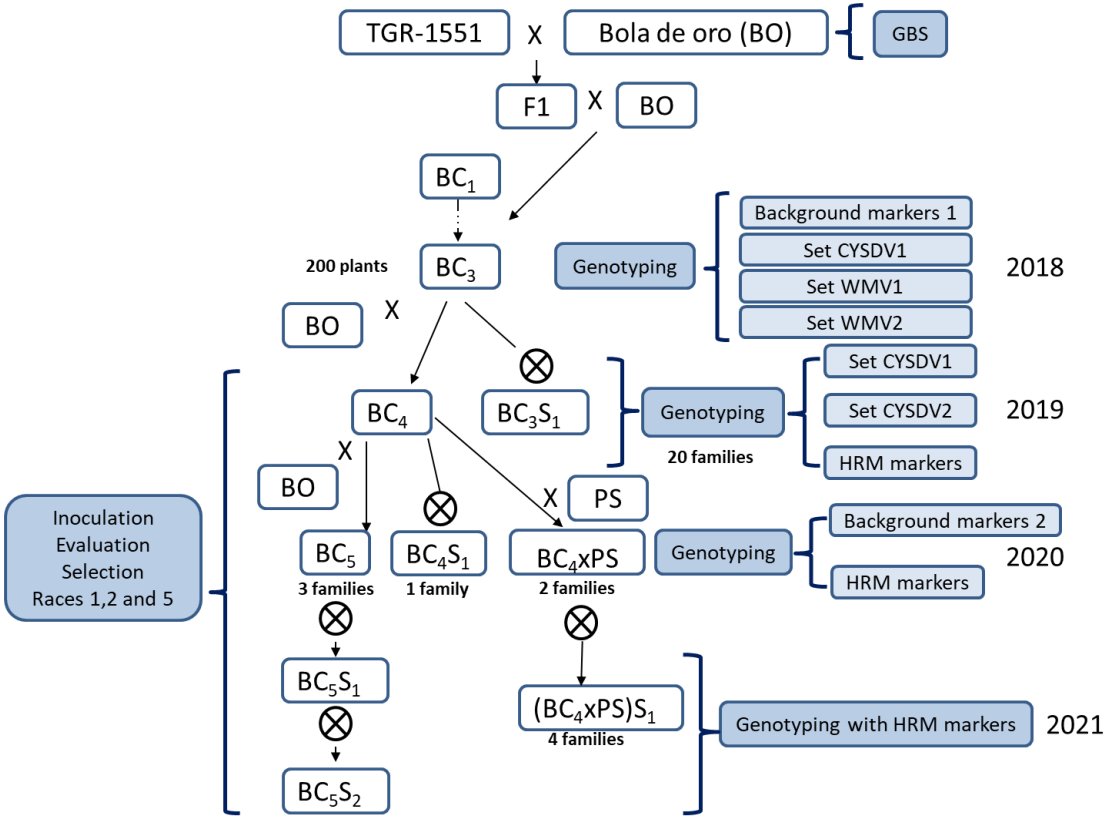
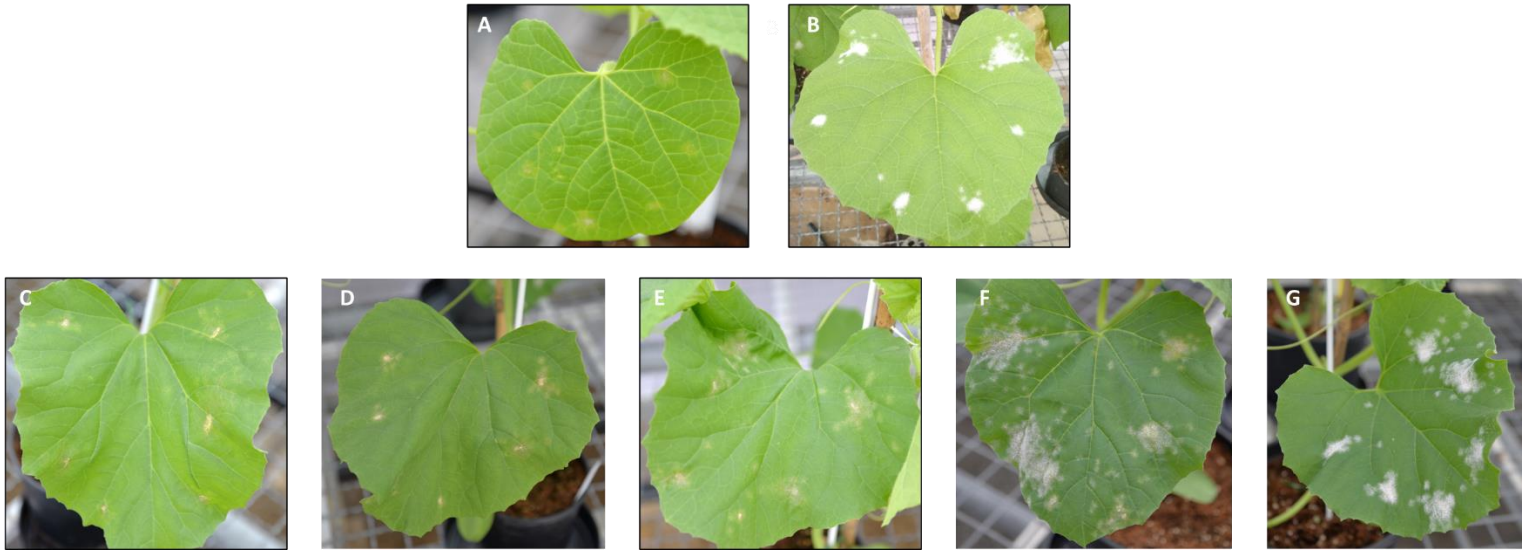
Supplementary materials

Supplementary Materials: The supporting information can be downloaded at: https://www.mdpi. com/article/10.3390/ijms232012553/s1

Supplementary Figure S1. Plant material generated and methodology used.



Supplementary Figure S2: Phenotypic classes established according to the level of sporulation of *Podosphaera xanthii* on melon leaves 12 days after inoculation. A) resistant parental line TGR-1551, class 0; B) susceptible parental line ´Bola de oro´, class 3; C-E) symthoms segregation observed within a BC3S1 family: C) class 1, D) class 1.5, E) class 2, F) class 2.5 and G) class 3.



Supplementary Table S1. Markers sets Background1 and Background2 used to genotyoe the studied families. The SNPs of the Background1 set have been retrieves from Perpiña *et al*., 2016. The physical position of the SNPs in versions v.3.6.1 and v.4.0 of the melon genome (available at https://melonomics.net/) is indicated. The SNPs (in brackets) and flanking sequences are included.

Due to its extent, Supplementary Table S1 can be found at https://www.mdpi.com/article/10.3390/ijms232012553/s1.

Supplementary Table S2. Marker sets (WMV1, WMV2, CYSDV1 and CYSDV2) used to genotype the studied families. The SNPs have been retrieved from Perez-de-Castro *et al*. (2019) and Pérez-de-Castro *et al*. (2020). The physical position in versions v.3.6.1. and v.4.0 of the melon genome (available at https://www.melonomics.net/) is indicated. The SNP (in brackets) and flanking sequences are included.

Marker set	Chromosome	Marker name	Genomic position (pb) v3.6.1.	Genomic position (bp) v.4.0	SNP and flanking sequences
CYSDV1[a]	5	*cysdv10B*	6412266	6298039	CCTTTCTTTCCAATCTATATTCACAACATTCACAGAGACAAACATGGCAGCGACACTGGA ACCTATGACACTGAAGGAAGGTA[T/C]GTAACTTTTCTTACTTCTTTGCCATTTGAAAGTG TACTGGTATCATATTGAGTAATGTAGGATAAGATGTAACAAACGAGCAGGT
CYSDV1	5	*cysdv11*	9593263	9494514	TCAGGATGAACAAGGTGCAATGCATTAATAGCAGCAGAAAGGCTATTCAATCGCTCCA ACACCAATGATGAACTGAACTG[G/A]TTATCTCCCAGAGCTCCTTCCGTTCTCAAACGAG CCGAATAAAGGTAGATATAACTTGCTGCTTTCTGCCAATTATGTCGGTGTA
WMV1[b]	5	*b5wmv2*	14945725	15026076	TCTTGGAGTACTTGCAGCRTTTTAAAGAGCTATYGGCGACACMCTAGACATGGCCGAAG ATGTGCTGGTAAACATGTTCACAAATGGGCTTGACCTAGT[C/T]AGCTGTATGGAGGTTT TCTCCATGAGAATTGTGGGCCTCAAGGATATGATGGACGCAGCCCGGTTAGCAGAGGA AAAACTTGAAACGGCCTGCACGGCCC
CYSDV1	5	*cysdv14*[1]	17265147	17216441	GATAGTTTCAACAACAAGAATTATTATAAATTACATAATGAACAATCGTAAGTCCACTA AATCCTAGTAGATGGCAGCCATTAATA[G/A]GCATGATTGTGTCCCTCAAGTTCATCACA TATCATGAAAGAGCCTCCTAACGTGATCAACATTTGCAGGCATTAATGTGATTGGGCA
WMV1	5	*b5wmv3*	20677985	19968717	AGACTTCAGCTCCAAGAAAATCTCTTTTGCTAAAGTAGGCATTCTTCTTCGGCTCAATTTT TTAATCACTTGAGAATTGTTGATATTAAAACCGTAGTC[G/A]ACAGAACTTCCTTCATTA TGCTGCTGGAATATCTAGCATAGGTACATTGCATAAAGAATAAGCAAAAATTAAGGTAG TGAAACTCTTTAAAACAAACATC
CYSDV1	5	*cysdv17*	22651076	22544163	CGACCATTACGTCTTCGCCGCTGCAGGTGTTCGTGCATCATTTCCGTCGCATCCGACAAG TCGAATGTCCGATTCTTCAA[T/C]CGCCACTCTCGTCCGCGGTTTCGACTGGTTGCGCCGC TGCAGCCGCGTCTCCCCCACTCGTCGGAGTTGGCCTTCTTTT
CYSDV1	5	*cysdv18*	24296585	24125662	GAAAATTTTCAATTAACATGTCATACCTTTTGATTCCTGCTGGTGCAGCATTATGAACTAT GTGATGATGCGCTGGATC[A/T]AGTGCATCACTTATTGAGTCGGGCATTATCGATCCAAG AAAACCACCTGACCTTCTGCTGCTTCTGAGGAAAAAGGAA
CYSDV1	5	*cysdv19*	24365016	24192280	TAATGAATGAGCAAAAGCAGCCACTCCGTTCGGAGCAAAAACATAGGATTTTAGTCTTT CAACGTGGCCCCGCATCTTCACAGC[T/C]GACGTACGAAGTGCAGTATACTCTGAAGCC CTACGAGCAGCTGCACAAAGATGAGTTTGAGCTTCCTCTCTCGCTTCATGC
CYSDV1	5	*cysdv21*	24605826	24427738	GTAGGTACCTGAAGCGAGATCGGCGACGCAAACGTCCTGAAGCAAATCGGGATCGGCA GCAGTGACAACAAAGAAGAAGA[T/G]GGCAAGAGAAGCAGTAAACAGAGCAGAGGA ATGATGAGGCAGCTTCATGATTGTGAAAAAGGTTTGGTGAAGAAGAG
CYSDV1	5	*cysdv22*	24613028	24434940	GATCGATTTCCTTGCTGCATCTACATTCAGTTTATAAAATGCAGATTCACCTTTGATATAA AAGCAATCCACCAGTCTAATTTG[A/G]AGCTGCCATCGAAGTTGATCACTATAGATAAT GAGTTCTTAAGGATAACACATATTTTTCCAAATTAATCTAGCATAAAACTT
CYSDV2[a]	5	*cysdv40*	24652307	24474795	CCCTCCACGCTATTCCGTGAACAAAGGGAAGCAGCAATTACAGATTCAACAGCCTTCTC ACTATCTGC[C/A]WATCCTTTCGTTGCACCAACTTCAAGACAATGTTTGAGGCGGTCATC ATGGTCRGCTGAATCACCCA
CYSDV2	5	*cysdv42*	24792185	24608464	TACGCTTATTGAAATATCACAACATAGGATCATAAGTTTGTTCATGACTTCAATATGCAA TGAATTGATT[T/C]TAATGGCTTGACTACACCTTAGAACAAGTCTCTTTGATGCCACCAAT GGACCATTCTCTCTGAAGAT
CYSDV2	5	*cysdv43*	24864545	24678113	CTGGTCCGCGGTGCAAAGAGAGACGAATGTGGGACTGAGATGGTCGGTGAATCTACAG GCGTGACG[A/G]AAAAGACGGACAAGGAGGTGCGACTTGAAAGGATGGCTGCCAACG AACCGGTGGTGAAGAG
CYSDV2	5	*cysdv44*	24890589	24704705	CAGCGACCCCAAAAATACGTGAGTTTTATCCATTATATGTAATATGTATTATGTATTCTAC TTCTCTGC[C/G]TTGCATTATCCTTCTCCGTAGAATATCTAGATCTACTTTATTCCTATAAA AAAATTGTTCCTGTGATTTG
CYSDV2	5	*cysdv45*	24945626	24761527	TTTTCTTCCATGTTTCTTCTCGTAATACCATGTGGGTTCTATATCTTGGGCATTGTCTCATG CACTTCCTA[A/G]TTCCATTTGTCAGGCTGGAGCAGTTGGGTTAATATTTCAGGCTGCACT TTTGACAGTTGCTGTTGC
CYSDV2	5	*cysdv46*	24962187	24778081	TGAGGGTCACCTTAAGTAATGTCATAGCTAACCAGGATAGAAGGCAGACAGAAAAAGC TTCAGCAGC[A/T]GATTACTTGAAGCAATATGATGCGGTTAACCGTCTATCAAGAAATTT TCAGACTGAACAGAGTTTT
CYSDV2	5	*cysdv48*	25026788	24842569	GTCACTTGAAAATGCTTCAAATCCACTACCAAACATTCATCAAAGCTTCCAACTAATACT CCAAATGCCA[A/T]CAAATGCCTTTTTCAATTCAATATTTCTATCACCTCCAATTCCATGG AACAAAACAGTTTTAAAAATG

Supplementary Table S2. Continuation

Marker set	Chromosome	Marker name	Genomic position (pb) v3.6.1.	Genomic position (bp) v.4.0	SNP and flanking sequences
CYSDV2	5	*cysdv49*	25027045	24842825	CATCATCTAAACTCGGAGTTCTCATGATCTACAGATTCATCCGACGGCGATGATTCATAG TGGTATGA[C/T]TCTTCAAAATCTTCCAAGTTGTTGTATATAGGAGCATCGTAAACATGT GAGCCGTCAGAGGAAGCTT
WMV2[b]	5	*SNP25*	18355735	24898321	ACCTACTTATTATGTTAATATCTACGTTTTCCAGATTCATTTTCTTTTTATTTGTATTATTCT AAATCTCCAGAACTGCTTAGCTGCTCTGGTTTTTGGG[G/A]ATTTTWGGGGGCTCGATCT GGTGGGTTTACGGTTAAATTTTGCAGCTTTTCGAGGTCCTTTTCGGCTTCCATTTTGTCGG AAGTTACAAAATGTTTTTGG
WMV2	5	*SNP26*	25081882	25045765	AAGTGAAAACTTAAGATAATAACTACATACAAATCCGCAACAAACTAACTAATAGGCT GCAGTGAAATAGTTTGCTGGTGGCTTTTAGTTTTAAGAAAAT[G/A]GTGAACATCCAGA GGAGAAAATTTAAGTGGATCATTCCATTGCTAAGAAAATAAAGGCAGCCCAAATTTCAA ACAGACAGTTATGGAAAACAAATTCAAG
CYSDV2	5	*cysdv50*	25236105	25052002	TTCATCCAAAAATCCAAAGTCATCTAATAATCCGTTTGTTTGTTCAATTTGAGTCAATGGT TC[A/G]GCATTGTATGCTTGGAAAGAACCAGTAACACTTTTTCCTATTTCCATGGGCTGA TTGAAAGAATTGTGCT
CYSDV2	5	*cysdv51*	25314484	25132216	AATGAGAAACATACTGTTGATTTTGCTTAAGAAATAGCCGGCAGCTCGGATTTATGTTGC AATTTG[G/A]CGGATCTACGCTTTTGAACTGAAAATATGCTCAATCGATACCTCAAACTT CATAGTTTCCTCGTTGACTT
WMV1	5	*b5wmv4*	25314558	25132292	GCTCGGATTTATGTTGCAATTTGRCGGATCTACGCTTTTGAACTGAAAATATGCTCAATC GATACCTCAAACTTCATAGTTTCCTCGTTGACTTTTCTT[A/G]TTTTTCTTGTATTTTTTTTTT AATGAACTCTTTTTGGATCAGAGCTCGATGGTGGCAGCGCTAGATCATACGGAGAAGAT TCTTCAGAATCGCCGGTGAG
WMV1	5	*b5wmv4A*	25326396	25144133	CCCTTTTTCAATCCCTTCCATTGTCATCATCCTCCTCTAATTCTTCTTCTTYTAAACAGTTTC CTCCAAGATTCACACCAGAATATCAACTACTCTGCC[G/C]TAGARCTCGCCCTAATGGCT CTTCAAAAGAGGGAACATCAACTTGGCTGCTTCAAGTTGAAGAGAGGTCAAAAGGGTA TCTTCCAAATTGGAGAAAAGTA
CYSDV2	5	*cysdv53*	25326351	25144085	TTCTAATTATATCATCCAAAACCCCTTTTTCAATCCCTTCCATTGTCATCATCCTCCTCTAA TTCTTCTTCTT[C/T]TAAACAGTTTCCTCCAAGATTCACACCAGAATATCAACTACTCTGC CGTAGAGCTCGCCCTAATGGC
CYSDV2	5	*cysdv54*	25392541	25210212	GGCTCAGCAGCTGCTGGGATATGTCGGTGGTCGAAGAGGCAACGGGTGGTTGGTTTTGG AGCAGGG[G/A]TGGCAGTGCAACGGCGGGTGCGTGGTGGTAGTGATGGTGAAGGTTGA GTGTATTTCTAGGTTGC
CYSDV2	5	*cysdv55*	25392903	25210573	TTGTTCCTTTGCTGCTGCTGGTGCCTCCCGTCCCCACACTCGGTACACCAGATTCATCACC AGTCGCCTT[C/T]TTGAGGCATGACTTGAGCTGAACTGCTGGGAGTGGTGGAAGAGGTG GTTGATGTACCACAGTCG
CYSDV2	5	*cysdv56*	25415551	25233221	GCTGTGGCCAGGTTTATTTATTGGTTCATAGTCATTTAGTGAAGCCCAAAGATGCTCAGG GGATTGATA[T/C]TGAGTGCACGGAAAATGCCAATGTGAAATCTGTGGTGAATGAGCTG CATAGTGATAAAGAATTG
CYSDV2	5	*cysdv57*	25526168	25341873	GCATTTCTAGTAAAGGAACCATGGATTCCGGGATTTCTTTCTCTCAGCCATCTTCCTTCTG GATGTGTTA[T/C]TGTATGGATAGTGAAATCACTTCCTTCTTCATCATAGATTTTGATAAA TGCTGGTAAGAACATCGTG
CYSDV2	5	*cysdv58*	25540372	25356079	TTTCTTTGCAGCTGATATTTTGCTGTTCGTTGAGAACAATGATGAATTTATCAGGAATATG CATATTGCT[A/T]TGCATCTCTTTGAATGTGCTACTGGTTTAAACCTAAAACAGGGCTAA ATCTACCATCTCCCCGATTA
CYSDV2	5	*cysdv59*	25619503	25435244	TTTTGCTGTTTCTTTTGTTCCATATTGACCAAAGAAGAGCAGCACCCCACTAAAATTGATG ATATTTTC[C/T]TCATTTGTTTGTTTGAGTGAGCACATAGCACTTTCAAGGCATTTGATATT GCTTTTGGTTTGCAAAAT
WMV1	5	*b5wmv5*	25895289	25694699	GTATACCTTTGAGCAGCTCAAACTATCCAAATTGCAAATATCACTTGATCCAGGATGTGT TGGAGTTTTACTGTCAAAAGGAAGGAAAAGTCAAAAACC[T/C]TAATAATTGTAAGCAG GTAAATGCAAAGTCTAGTCACTGATTTTGTGGATTCACCTCATTTCAAGTTCTGAAGTAC ATTGTGGTGAATAGATTTTCAAGG
CYSDV2	5	*cysdv60*	25943991	25744140	AAAAGTGTGTCTTCATTCACTGCAAATGATGGAGGATTGAGTGTGAGGTTTTATGTGGCA GCAATAAG[G/A]GAAATAACGTCTATGGATAAGGGAGAACTGTAATTGTGAGATTGCA AAAGAACCCAGCAAAGAT
CYSDV2	5	*cysdv61*	25956650	25756801	AGAACCTGATAAAGCAAGCAGAGCAGCTGATAGGGCAACGCAAAACGCTGCTGATGAT CCCAAGCC[G/A]GATCCAAGAGGAAGCTCAGAAGTGATGGCCACCTCAACAGGCACAA ATCTGAAACACAAGGGAGC
CYSDV2	5	*cysdv62*	25975889	25776015	CCATCTCGGGCTGGTCGAAGGTCAGAAACATTTTACCTCCATGACCAAGGTTTACGGTAT TGAGCCTAC[C/T]ATGGAGCATTATGGTTGCATGATTGACCTTTTGAGTCGAGCAGGCCA CTCCGAAGAGGCCGAGGA
CYSDV2	5	*cysdv63*[1]	25982529	25782654	GAAGATGTTGAAAGGTTTAAGAGGCTAACAGGACTGGATGACTTCTTTTTGGATAGAGA TGCAGCCA[C/G]ATCACACAATTCGTCAGCTCGTAAAGCTTCACTCAATGTTGAAGCTAC AGAGAATAGATCGAATAAAG
CYSDV2	5	*cysdv65*[1]	26629653	26408895	CAGTCATTGAGATCTCTAAACAAATCAGAGGTAATAACGGTGAAAAAACCAACTGCAG CCAAGAGGGC[G/A]TACAAGAACGGCTTGTTTTCGGATACACTCTGGTTAAAGGGATGA CCCATGTAGTTTACGGCAAA
CYSDV2	5	*cysdv68*	26682391	26461677	CCCTCTCCAACAGCTCAATGATCTCAACAGCTCTCCTTACCTCTCGGTTTGCTCGTAACGC AGCCACAAC[G/A]TTAATAATCGTTCTTTTTGTCTTGGCATCAATTTAACTTTTACAACCA TTTCTTTTAGCAATTCTACTG
CYSDV2	5	*cysdv69*	26688074	26467357	ACAAAATGCAGAATGATTATAAAGTAACTGTAACCAGCAGCCATTTATATAGGCTGACA TAAACAACC[T/C]AAGGGAAAAGAGGCTCTCGACAAAAACAAAGAACTAACTAACAG CTTATTAAATAAAGCTGTAAA

Supplementary Table S2. Continuation

Marker set	Chromosome	Marker name	Genomic position (pb) v3.6.1.	Genomic position (bp) v.4.0	SNP and flanking sequences
CYSDV1	5	*cysdv24*	26766636	26547204	TAATTGCCAAAGCAGTTTTAGCAGCAAGAGACCGAGAAATTTTTCCTTTGAGCTTTGGAG CTGCCTGACCAATTAGGGATGC[A/G]TGGTAGATAAGACCATATTTGGGAGTTGCATGC TTTGTTTTCAAAGCTCTGAACAAAGCTTTCTCAGCTCCAAGAATTTGCACTG
WMV1	5	*b5wmv7*	26816701	26592732	AGTAAAAAAGAAAGGTTGGGGGTAAAACGCAGCTTTCGAGAGGCAAGGCAGCGTAAA TAAAAATAGAAAAATTAAAATAAGAAAATGCACACATTTTTT[T/G]GATTTTCTTCAGCA ATTTGCACTTGAACGCTAAAAGAAGTATTCGACAAAAAAGGTTATTTGTTGCAGCGATA ACACAAAACCTTAAGCTTAGCTTTACA
WMV1	5	*b5wmv7C*	26940315	26715854	ATCAACTGGCTCCCTGCAGCACGTTGATTGTTCAATAACCAAAGACAAATGTACAATCC AAGTCTGTGACAAGGTTTGTTCAACTCCTGGTTACACTAG[G/C]GCATGGCAGGGAATA CAATGCAGCTTATTCCAAGATGACCAAGTGGCAAGAAGCGATTCCTCAGATCAGAATGC AACTGCTGGSGCATCAAGCCATACCT
CYSDV1	5	*cysdv23*	26976581	26752698	CCATTCTCCATCGTCGTTTGTGAGTTTTGGGATTAACCGAGCATTCTTCATAGTCAATCTC AACTATGTCATCATTTTTTCTATA[C/A]CTCAACAATGCCATTGATACAAACAGAGAATG CTGCTTTGATTTTGGTGAAAACAAAGGATGATCGAAAGAGAGAAACAGAAA
CYSDV1	5	*cysdv25*	26993475	26769546	GCATATTGAAGCTGATCATGGTGTTGTTATACATAATAACTGAGTGGTTGGACTGGATGA AGCCCATACCACCCATATCAGC[T/A]GCCAGATTGAAGACATGGTCAACTTTCTCAGTCA CCTTCAAACAATTATCCATCACTCTGAGATCAACAAGATGAAATTCATGGC
CYSDV1	5	*cysdv26*	27170637	26940168	CTCCAACCCTTCTCTTCTCCTCCAAACCACCATCCAGAAAAACACCCTCCTCCATGTGGC AGCTGCCTTCAATCAGAA[G/A]AGCATTGCCGAAGAGATCACCAACCGTCATCCTCCGA TTCTGTACGCGACAAACTCAAAAGAGG
CYSDV1	5	*cysdv27*	27188971	26957159	GTTGCTGATGAGATGGAGGAGAGAAATGATAAGAAAAGGTGACGATTTGGGAATGACC CCTCTCCACTTTGCTGCTTT[A/C]TATGGAAGGATTGAAGTAGTTAAGTTGTTTGTTGAGT ATGATAGCAGTGCAATTTACGTGA
WMV1	5	*b5wmv8*	27194925	26963176	GTGAATCTCAATGGCGATACGGCTTTGCATTGTGCTGCGAGAATTGGGAGTTTGATATGT GTGGAGAAGATTGTGGAAGCGGAGCCGGAGCTTTGCGGC[G/T]TCGTGAATAATAACG GGGAATCGCCGTTGTACTTAGCGGTGGCTGCCGGATTTTGGGAAGTTCCTCAGGTCATTA TTCGTAAGGCGGAATTGTTGGCTTC
CYSDV1	5	*cysdv28*	27353196	27118062	CCCATTATGTTGTTTGCTAAGGGCAGCCCTACAACTTTAGGGTTAACATTTCCAAGTCTAC AAATTCAATAGCCTTCTGTTTC[T/G]TAAAACACTGCTGCTACCTAATGAGATTTCCCTCT TTTCATTGTTCAAAAATGGAAAAATAAATGAATTATTACTTTTGAATCC
WMV1	5	*b5wmv9*	27509294	27273256	AAAGGAAGACGAGTTCTTGATCATAGGTAGCGATGGAATATGGGACGTCTTTACAAGTC AAAACGCCATTGATTTTGCTCGGAGGAAGCTCCAAGAACA[C/T]AATGATGTGAAAATT TGCTGCAAGGAAATAGTAGAGGAAGCCATAAAGAGAGGAGCAACAGACAATTTAACA GTAGTATTGGTTAGCTTTCATTTGGAGC
WMV1	5	*b5wmv10*	27698241	27464146	CCAGCTAAAGAAGTTGGAAGAGCAGCTGGTGTAGGCTGAAATGGCTACAAACACACAA AGCAGACCAGCATTCAAACTCCATGGGCCAAGAAACAATCT[A/G]ATAGCTTGAAAATT TATAAAACAAACAATGTCCTAAACTTACACCATGCGCACTCAAGGGAGGAAAGGCTGC
CYSDV1/WMV2	5	*cysdv29/SNP29*	27772725	27538308	GTCTAAGACCAAATGTTGAATGGAATGGCATGATTGTATATATCCCATTTGTAAGTTCCT TTAGAGTTGGTCACAGAAACTAA[C/T]CTCCATAAATTTGAGAATGCTGCTTCTCCCCAT CATCCTCATCACACAATAACCAAAAACATCTGGAATTTTCACTAATTTTTTTA
WMV1	5	*b5wmv11*[1]	27806146	27570154	TTGCTATCAAAAACTCCCATATCCTGAATGGCCAATTTTTGGTTAATAATTGCATAGCTCG TACATTAAGCAGTATTGCAAAAGACGTTTTAACTGTGG[A/G]ATCGGATATTGTTAGAA AAATTCGGGATTGTGTAAAGAATGTAAAGTTGTCCGAGTCTAATGAGGCAAAGTTT
CYSDV1	5	*cysdv30B*	27806568	27570488	GGAGGCAAGTTGAGACTCTTTGCTCGTGTTTGAAAGTTCTCTTTGATGCTGCAAGCATTCT CAGCACCATAAACAACCCAACCGT[A/G]ATTACCTTTTTTCACGAAGTATGGAGGATTC ACTTGGAGCTTGCCCGTGCACTCACGAGTGAAGATCCGTTCACTTGCAGCCTCACT

[1]: markers adapted to detection by HRM
[a]: markers retrieved from Pérez-de-Castro et al. (2020)
[b]: markers retrieved from Pérez-de-Castro et al. (2019)

Supplementary Table S3. MELOs annotated in the candidate region of chromosome 5, according to the last version of the genome annotation (v.4.0 available at https://www.melonomics.net/). The position of the Melos and their description, molecular function, biological procces and cellular component are included when available.

Gene name	Position in chromosome 5 (start..end bp)	Description	Molecular function	Biological process	Cellular component
MELO3C004308.2.1	26055613..26059525	Photosystem II reaction center PsbP family protein	Calcium ion binding	Photosynthesis	extrinsic component of membrane/ Photosystem II oxygen evolving complex
MELO3C004309.2.1	26060336..26066739	TMV resistance protein N-like	ADP binding / Hydrolase activity	Defense response / Signal transduction	
MELO3C031551.2.1	26071210..26071521	LINE RNAaseH			
MELO3C004297.2.1	25990708..26007080	Branched-chain-amino-acid aminotransferase-like protein	Transaminase activity		
MELO3C004301.2.1	26027670..26034392	TMV resistance protein N-like isoform X1	ADP binding / Hydrolase activity	Signal transduction	
MELO3C004303.2.1	26036017..26040632	TMV resistance protein N-like	ADP binding / Hydrolase activity	Defense response / Signal transduction	
MELO3C031552.2.1	26040008..26040909	TMV resistance protein N-like	ADP binding / Hydrolase activity	Defense response / Signal transduction	
MELO3C004302.2.1	26036022..26036650	TMV resistance protein N-like isoform X1	ADP binding / Hydrolase activity	Signal transduction	
MELO3C004307.2.1	26051704..26054581	UPF0603 protein, chloroplastic	Acid phospatasse activity / mRNA binding	Photosystem II repair	Chloroplast / Nucleus
MELO3C031553.2.1	26070542..26072055	Ulp1 protease family, carboxy-terminal domain protein	Cysteine-type peptidase activity		
MELO3C004314.2.1	26119787..26121002	FANTASTIC four-like protein (DUF3049)			
MELO3C004315.2.1	26134314..26138449	receptor-like cytosolic serine/threonine-protein kinase RBK2	ATP binding / GTPase binding / Protein serine kinase activity / protein threonine kinase activity		Nucleus/ Cytoplasm
MELO3C004316.2.1	26140149..26142350	Prefoldin chaperone subunit family protein, putative	Chaperone binding / Unfolded protein binding	Chaperone-mediated protein complex assembly / Protein folding	Cytoplasm
MELO3C027615.2.1	26149781..26151117	Vat protein			
MELO3C004318.2.1	26166971..26168794	Vat protein			
MELO3C027385.2.1	26163815..26165513	Vat protein			
MELO3C000062.2.1	26162861..26163728	Vat protein			
MELO3C004311.2.1	26076967..26095083	TMV resistance protein N-like	ADP binding / Hydrolase activity	Defense response / Signal transduction	
MELO3C004313.2.1	26106563..26109785	TMV resistance protein N-like	ADP binding / Hydrolase activity	Defense response / Signal transduction	
MELO3C031554.2.1	26192370..26193423	Vat protein			
MELO3C031326.2.1	26194626..26195639	Vat protein			
MELO3C004319.2.1	26190064..26191780	Nucleotide binding site-leucine rich repeat protein	ADP binding		
MELO3C031555.2.1	26195989..26198249				
MELO3C004320.2.1	26203645..26205643	Disease resistance protein	ADP binding / ATP binding / Nucleotide binding	Cellular response to SA / Defense response / plant-type hypersensitive response/ positive regulation of defense response to virus / Response to wounding	Plasma membrane / Plastid
MELO3C004317.2.1	26204964..26206644	Vat protein			
MELO3C004321.2.1	26225697..26233806	Vat protein			
MELO3C031556.2.1	26263124..26265242	Disease resistance protein	ADP binding / ATP binding / Nucleotide binding	Cellular response to SA / Defense response / plant-type hypersensitive response/ positive regulation of defense response to virus / Response to wounding	Plasma membrane / Plastid
MELO3C004323.2.1	26261720..26262645	Vat protein	ADP binding		
MELO3C031325.2.1	26262518..26262960	Nucleotide binding site-leucine rich repeat protein			
MELO3C031324.2.1	26273595..26284104	Vat protein			
MELO3C031799.2.1	26216755..26216988				
MELO3C031329.2.1	26241868..26242954				
MELO3C028480.2.1	26268352..26268762	gamma-aminobutyrate transaminase POP2, mitochondrial-like			Mitochondrion / Cytosol / Golgi apparatus / Vacuolar membrane
MELO3C004329.2.1	26298777..26308596	Terpene cyclase/mutase family member	Beta-amyrin synthase activity / Lanosterol synthase activity	Triterpenoid biosynthetics process	Lipid droplet

Supplementary Table S4. Predicted impact of the detected SNPs

Gene name	Chromosome		GeneId	variants_impact_HIGH	variants_impact_LOW	variants_impact_MODERATE	variants_impact_MODIFIER	variants_effect_3_prime_UTR_variant	variants_effect_5_prime_UTR_premature_start_codon_gain_variant	variants_effect_5_prime_UTR_variant	variants_effect_downstream_gene_variant	variants_effect_frameshift_variant	variants_effect_initiator_codon_variant	variants_effect_intron_variant	variants_effect_missense_variant	variants_effect_non_coding_transcript	variants_effect_splice_acceptor_variant	variants_effect_splice_donor_variant	variants_effect_splice_region_variant	variants_effect_start_lost	variants_effect_stop_gained	variants_effect_stop_lost	variants_effect_stop_retained_variant	variants_effect_synonymous_variant	variants_effect_upstream_gene_variant
MELO3C004297.2	5	initiation codon	Branched-chain-amino-acid aminotransferase-like protein	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
MELO3C004311.2	5	2 intron	TMV resistance protein N-like	0	0	0	2	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0
MELO3C004297.2	5	downstream , 4 intron	Branched-chain-amino-acid aminotransferase-like protein	0	0	0	5	0	0	0	1	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0
MELO3C002520.2	12	start loss	(3S,6E)-nerolidol synthase 1-like	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
MELO3C002515.2	12	3 UTR	Eukaryotic translation initiation factor-like protein	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
MELO3C002514.2	12	stop gained	BTB/POZ domain-containing protein At3g22104	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
MELO3C002536.2	12	missense & sinonimus	Serine/threonine-protein kinase	0	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0
MELO3C002504.2	12	sinonimus	cysteine-rich receptor-like protein kinase 28	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
MELO3C002532.2	12	sinonimus	Serine/threonine-protein kinase	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
MELO3C002534.2	12	downstream	Serine/threonine-protein kinase	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
MELO3C002526.2	12	intron	Protein DETOXIFICATION	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0
MELO3C035528.2	12	down y upstream	Serine/threonine-protein kinase	0	0	0	5	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4
MELO3C002530.2	12	missense & sinonimus	Serine/threonine-protein kinase	0	2	2	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	2	0
MELO3C035727.2	12	intron	Unknown protein	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
MELO3C002537.2	12	sinonimus	Serine/threonine-protein kinase	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
MELO3C002541.2	12	missense & sinonimus	receptor-like serine/threonine-protein kinase SD1-8	0	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0
MELO3C002524.2	12	intron	Serine/threonine-protein kinase	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
MELO3C002527.2	12	downstream	Serine/threonine-protein kinase	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
MELO3C002529.2	12	upstream	Serine/threonine-protein kinase	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4
MELO3C002538.2	12	down & upstream	receptor-like serine/threonine-protein kinase SD1-8	0	0	0	3	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
MELO3C002545.2	12	downstream	purine permease 3-like	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
MELO3C002546.2	12	missense	Serine/threonine-protein kinase	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0

Supplementary Table S5. Molecular markers used to narrow the candidate interval in chromosome 12. Primers sequences are provided for those markers that have been addapted to HRM. Gene loci and their descriptions are indicated according to the last version of the genome annotation (v.4.0, available at https://www.melonomics.net/). SNPs (in brackets) and flanking sequences are provided.

Marker name	Chr[a]	Genomic position (bp) v.4.0	HRM forward primer sequence	HRM reverse primer sequence	Gene locus	Description v.4.0	SNP and flanking sequences
CMPSNP285	12	20421309	-	-	MELO3C002713.2.1	S-adenosyl-L-methionine-dependent methyltransferase	GAGGTTATCTTTGGTTTGATCATTTCTTCAGCAAAGGGGTGGATCTTGATAAACTTTAC[T/G]CTC CTTTGATTACCAAATTGGGTTATCGGAAGGTCAAGTGGGCGACAGCAAATAAGACCGA
Oidio 8	12	20920054	TTGAATGGATGGTGTATCGTG	CGGAATGAAGCATCACAGTC	MELO3C002658.2.1	Transcription initiation factor TFIID subunit 11, putative	CACTCGCAGTATCTATGTAGAGTGTCTTCTCCATCAGGAAACTTGCCATGTCAACCCCATCTTTAT TTGCTTGAATGGATGGTGTATCGTGAAAGTCAA[C/T]ATCACCTTTAGATTTGGAGATAGTGTCAA
Oidio 5	12	21470961	TTGCATAGGAGGACACATGG	GAGGGAAGAAGATCTCATCAG	MELO3C002603.2.1	Callose synthase 11	CTTCTTTAGGAACTACAAATGGCTGCCTTCTACAAGGAGACTGTGATGCTTCATTCCGGAAGGGA CTCGTCAGGTTTATTTGCAAGCTCTGTTGCCTGACTTAGAGCAAGGAGCAGCTCGTTGCATAGGA
Oidio4	12	21866445	TTTACTTTCGCGCTCTCCAG	CTGAAACACGGGCAGTTTTG	MELO3C002556.2.1	Heterogeneous nuclear ribonucleoprotein U-like protein 1	GGACACATGGCCAACGAATTACCCTAATACTCCA[A/G]TAATTGGGTGGAAGCTCCAAGAGATC GAAATCTCTATCGCTGATGAGATCTTCTTCCCTCATAGTAATGAGAATCTCATTCCAAATCAAAGC TGCGCGGCAACACTCAGTTCGTTTACTTTCGCGCTCTCCAGATTTGCAATGGCGTCCAAACGCCA
Oidio3	12	21870304	TGTTTTACCAATTACCCATACGC	CATACCTCGTTGACCTGAAAAAG	MELO3C002556.2.1	UTR/intron ****heterogeneous nuclear ribonucleoprotein U-like protein 1	CCGTTCTCAAGAACACGAATGCGAGGTGAAGGAA[T/C]CCAAAACTGCCCGTGTTTCAGATGCTT TTGAGTTGAAGAAGCAGCAACCAGTAGTGCTCAACCCTGCCGACTGCGACCTGGGTATTTCTTCT GCTAAGATGAGTAGAAAAATACTCTGTTCAATCTGCTTATCTTTTCATGTTTTACCAATTACCCATA
12m12	12	21923406	-	-	MELO3C002550.2.1	Flowering locus T/terminal flower 1-like protein	CGCTATATGAATTCATTGAGCTTGTTAGTAAC[A/C]GTTGCTGCATCTTCACTTTATTTTCACATCT CTAAATCATTCTTTTTCAGGTCAACGAGGTATGCCAGATGCTGGACGATATGGAGTTCCAAGATTT TGCATCCAAGAAACGCTGATATATATAACATATCCATATTCCGTWCCACACTTTGTATGTT/AAAT ACCATACCCTCAACTTTTTTTCTACTTAGCAAATTTAGGCCTTCCCATGCTGCTGT
Oidio2	12	22251703	AGGTCCAATGTGCCGAATAG	CCCTTCTCACCAATAACTGTCC	MELO3C002504.2.1	Cysteine-rich receptor-like protein kinase 28	AAACCTAGCGTCCGAAACATTATTGGTCACGGGCAGCACCACAAAGCGAGTGGGGGAAAGGTC CAATGTGCCGAATAGAGAGCGGTCGGAGTAACGAAA[A/G]AAACAGTCTTCGTACCAAACAATG GCATTAGTTTGGGTGGGACAGTTATTGGTGAGAAGGGTTATTGAGTTGGTGAGGCAGCTTCTGC
Oidio1	12	22309972	AAATTAGCCATCTGGGTTGC	CGACAAGGCTCGATTCAAC	MELO3C002489.2.1	Cysteine-rich receptor-like protein kinase 10	TCTCCGCAATCTGTATTAGACAAATCGCCGGTACACTGAGCTAATGTGTAAATATTTCGATCTTGT GTGAAATTAGCCATCTGGGTTGCAAACAATTCA[T/C]CCATTGAAGAGGCTGCCAGGGCCGCGG
OidioC	12	22875711	TGTTGGAATATTTCTGATGCTG	ATCCGCAAACTCTCAAGACG	MELO3C002406.2.1	Bifunctional purine biosynthesis protein PurH	TGGCTCTCAACGTGGACATGACTAATTGGTTGAATCGAGCCTTGTCGCCATCTATATTAACCAGAT TGATAGCAAAGGTTAGTATAATTCTAACTCTGAATTTTTCTTGACATGTTTGTTTTCTGTTGGAATA TTTCTGATGCTGACTCTTTTTTGGCAGAATAA[T/C]TGCATGTTGGGTATGGGAAGCGGGCAACC
Oido B	12	22913643	TGCTGTCTATTCCTTTCAGC	GAGTGCAGTTGAAGCAGCAG	MELO3C002403.2.1/ MELO3C002402.2.1	Allantoate deiminase/C-type lectin receptor-like tyrosine-protein kinase At1g52310	AAATCGTCTTGAGAGTTTGCGGATAGCCTTGAGGAAAGCAGGGGATGAGGTGAAAGGAGCTGC TTCCATCTTGTCCACTGTCCAAATTTACCATTTGCTTCCATTTCACAATCTTAATCTGCTGTCTATTC CTTTCAGCATGATGCAAATGCCATAATTAGC[G/A]ATTCGGAGCTGAGCTCGCAACTGAAATCTG
Oidio A	12	22915510	GAAGACGGTTTCCCTCACTG	CACTGGTACCGCCTTTGTG	MELO3C002402.2.1	C-type lectin receptor-like tyrosine-protein kinase At1g52310	CTGCTTCAACTGCACTCAAAAAGATGGTAGGCGAGATTCAGGAGGAAGTACCTGTGTTAATGAG ATCAGGTAGAAGTCCACTGTATGTACCACCTGTTTTTGCATCTCCAAGAAGACGGTTTCCCTCACT GAAGTTTTTTGTCATCGATCTTAGCTCTTCTTTT[G/T]GTAAACACCCTCCACAAAGGCGGTACCAG
CMPSNP361	12	23000406	GAGCTCGATTTTGTGCTTCG	ACCCATAGCTTCCCTTCCTC	MELO3C002387.2.1	Chromophore lyase CRL, chloroplastic	TGCAGAAGCTGCAGGGTTAGATGGCTTCCGCGACCTTCTCCGCTTTTTGCTCCTCTTGTGGGCAA ATTCTTGGCTCTACTCTTTGGAGCTCGATTTTGTGCTTCGAATCATTTGGGGGAACCTG[A/G]AGA AGATTAGGGATGATGGGTGTTTGAGAACTGAGGAAGGGAAGCTATGGGTACCAATTCC

Supplementary Table S6. MELOs annotated in the candidate region of chromosome 12, according to the last version of the genome annotation (v.4.0 available at https://www.melonomics.net/). The position of the Melos and their description, molecular function, biological procces and cellular component are included when available.

Gene name	Position in chromosome 12 (start..end bp)	Description	Molecular function	Biological process	Cellular component
MELO3C002553.2	21898478..21901974	sugar carrier protein C-like	monosaccharide transmembrane transporter activity/ symporter activity	Sugar transport/ symport	
MELO3C002551.2	21911664..21913097	Transcription factor			
MELO3C002550.2	21920467..21924596	Flowering locus T/terminal flower 1-like protein	Phosphatidylethanolamina binding / Transcription coregulator activity	Cell differentiation / Regulation of flower development / Meristem determinacy / Regulation of stomatal movement.	Endoplasmic reticulum / Nucleus / Cytoplasm
MELO3C002552.2	21904739..21905323	Wound-responsive family protein	DNA replication independent nucleosome assembly		
MELO3C002549.2	21930564..21931178	Ribonuclease H domain-containing protein	Nucleic acid binding / Protein-disulfide reductase activity / RNA-DNA hybrid ribonnuclease activity		
MELO3C002548.2	21934022..21934534	B-box zinc finger protein 32-like	Dna-binding transcription factor activity / Zinc ion binding	Negative regulation od transcription, DNA-templated / Photomorphogenesis / Regulation of flower development / Reponse to chitin	Nucleus / Cytoplasm
MELO3C002546.2	21947518..21950780	Serine/threonine-protein kinase	ATP binding / GTPase binding / Protein serine/threonine kinase activity		Nucleus/ Cytoplasm
MELO3C002545.2	21952397..21953936	purine permease 3-like			
MELO3C002543.2	21964354..21968038	Serine/threonine-protein kinase	ATP binding / GTPase binding / Protein serine/threonine kinase activity		
MELO3C002542.2	21968776..21972156	Serine/threonine-protein kinase	ATP binding / GTPase binding / Protein serine/threonine kinase activity		
MELO3C002538.2	21981032..21989830	receptor-like serine/threonine-protein kinase SD1-8	ATP binding / Protein serine/threonine kinase activity / Carbohydrate binding / transmembrane receptor / Ubiquitin protein ligase binding	Protein phosphorylation / Recognition of pollen	Plasma membrane / Vacuole / Plasmodesma
MELO3C002541.2	21972870..21980428	receptor-like serine/threonine-protein kinase SD1-8	ATP binding / Protein serine/threonine kinase activity / Carbohydrate binding / transmembrane receptor / Ubiquitin protein ligase binding	Protein phosphorylation / Recognition of pollen	Plasma membrane / Vacuole / Plasmodesma
MELO3C002536.2	21977385..21984272	Serine/threonine-protein kinase	ATP binding / GTPase binding / Protein serine/threonine kinase activity		
MELO3C002537.2	21991983..21995487	Serine/threonine-protein kinase	ATP binding / GTPase binding / Protein serine/threonine kinase activity		
MELO3C002534.2	22009954..22013765	Serine/threonine-protein kinase	ATP binding / GTPase binding / Protein serine/threonine kinase activity		
MELO3C002532.2	22016954..22020180	Serine/threonine-protein kinase	ATP binding / GTPase binding / Protein serine/threonine kinase activity		
MELO3C002529.2	22028998..22030122	Serine/threonine-protein kinase	ATP binding / GTPase binding / Protein serine/threonine kinase activity		
MELO3C002527.2	22035451..22039235	Serine/threonine-protein kinase	ATP binding / GTPase binding / Protein serine/threonine kinase activity		
MELO3C002525.2	22052938..22053114	Unknown protein			
MELO3C002524.2	22054613..22067786	Serine/threonine-protein kinase	ATP binding / GTPase binding / Protein serine/threonine kinase activity		
MELO3C002521.2	22083111..22084488	At1g11440	phosphatidic acid binding/ protein serine/threonine kinase activity/ protein autophosphorylation	Protein autophosphorylation / Protein phosphorylation	
MELO3C001571.2	22087405..22087642	Unknown protein			
MELO3C002535.2	22003631..22007112	Serine/threonine-protein kinase	ATP binding / GTPase binding / Protein serine/threonine kinase activity		
MELO3C035727.2	22017146..22018120	Unknown protein			
MELO3C002530.2	22024773..22028148	Serine/threonine-protein kinase	ATP binding / GTPase binding / Protein serine/threonine kinase activity		
MELO3C035528.2	22021676..22023722	Serine/threonine-protein kinase	ATP binding / GTPase binding / Protein serine/threonine kinase activity		
MELO3C002528.2	22033421..22033717	Kinesin-like protein	protein binding/ microtubule based movement/ Golgi stack/thricome morphogenesis		
MELO3C002526.2	22040878..22045068	Protein DETOXIFICATION	Antiporter activity / Transmembrane transporter activity	Cadmium ion transport / Sequestering of metal ion	Plasma membrane
MELO3C002522.2	22069970..22073452	transcription factor bHLH112-like isoform X1	DNA bindinf transcription factor activity	Enhacen stress tolerance	Nucleus
MELO3C002520.2	22111113..22116528	(3S,6E)-nerolidol synthase 1-like	Magnesium ion binding / Terpene synthase activity	Diterpenoid biosynthetic process	Chloroplast
MELO3C002519.2	22117042..22117194	Unknown protein			
MELO3C002517.2	22140203..22146480	(3S,6E)-nerolidol synthase 1-like	Magnesium ion binding / Terpene synthase activity	Diterpenoid biosynthetic process	Chloroplast
MELO3C002516.2	22165544..22169211	(3S,6E)-nerolidol synthase 1-like	Magnesium ion binding / Terpene synthase activity	Diterpenoid biosynthetic process	Chloroplast
MELO3C035728.2	22176464..22178188	Unknown protein			
MELO3C035526.2	22183967..22188504	(3S,6E)-nerolidol synthase 1-like	Magnesium ion binding / Terpene synthase activity	Diterpenoid biosynthetic process	Chloroplast
MELO3C000787.2	22103865..22105366	Serine/threonine-protein phosphatase 7 long form-like protein	Metal ion binding / Protein serine-threonine phosphatase activity		Cytosol / Nucleus
MELO3C002515.2	22191857..22196696	Eukaryotic translation initiation factor-like protein	translational iniciation		
MELO3C002514.2	22197062..22199504	BTB/POZ domain-containing protein At3g22104	protein ubiquitination		
MELO3C002511.2	22210074..22211444	Transmembrane protein, putative (DUF1218)			Integral component of membrane

Supplementary Table S6. Continuation

Gene name	Position in chromosome 12 (start..end bp)	Description	Molecular function	Biological process	Cellular component
MELO3C002509.2	22217024..22222349	Kinesin-like protein	protein binding/ microtubule based movement/ Golgi stack/thricome morphogenesis	Microtubule-based movement	Kinesin complex / Microtubule
MELO3C002508.2	22223159..22225386	thioredoxin-like protein CXXS1	protein disulfide isomerase activity		Cytosol / Extracellular region
MELO3C002507.2	22226137..22229607	transcription factor ILR3-like			
MELO3C002501.2	22262412..22265400	cysteine-rich receptor-like protein kinase 26 isoform X1			
MELO3C002499.2	22277737..22278519	Cysteine-rich receptor-kinase-like protein	protein autophosphorylation / protein binding		Transmembrane signal receptor
MELO3C002513.2	22203933..22206026	Phosphosulfolactate synthase	Phosphosulfolactate synthase activity / Sulfopyruvate descarboxylase activity	Coenzyme M biosynthetic process	
MELO3C002512.2	22207646..22209486	exopolygalacturonase-like	Polygalacturonase activity	Carbohydrate metabolic process	Cell wall
MELO3C002510.2	22211483..22213022	oxygen-evolving enhancer protein 3-2, chloroplastic	Electron transfer activity / Calcium ion binding	Photosynthesis electron transport chain	Chloroplast / Cytosol / Apoplast
MELO3C002506.2	22229817..22238994	Receptor-like protein kinase	protein kinase activator/ protein binding		
MELO3C035729.2	22240996..22242173	cysteine-rich receptor-like protein kinase 28	ATP binding / Proteinserine/threonine kinase activity	Protein phosporylation	Plasma membrane
MELO3C002504.2	22242891..22252225	cysteine-rich receptor-like protein kinase 28	ATP binding / Proteinserine/threonine kinase activity	Protein phosporylation	Plasma membrane

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Chapter 5.

Breeding quality melons derived from the multirresistant accession TGR-1551.

Chapter 5. Breeding quality melons derived from the multirresistant accession TGR-1551.

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Abstract

Watermelon mosaic virus (WMV), cucurbit yellow stunting disorder virus (CYSDV) and powdery mildew (*Podosphaera xanthii* (Castagne) Braun) are three of the most important pathogens affecting melon (*Cucumis melo* L.). However, currently there are not commercial varieties resistant to these pathogens. The African accession TGR-1551 (*C.* melo, acidulus group) has been described as a reliable resistance source against all of them. The availability of molecular markers tightly linked to the resistance genes derived from TGR-1551 has allowed the development of a breeding program to introgress the three resistances into a yellow type genetic background. Molecular markers assisted selection was used in advanced backcross generations to select plants carrying the regions associated with the resistance. With the aim of recovering the quality traits of the recurrent parent, 'Bola de Oro', selection against the TGR-1551 background was performed for the rest of the genome, using previously developed markers uniformly distributed in the 12 chromosomes. The obtained BC_5S_2 pre-breeding lines were resistant to WMV, CYSDV and *Px,* and had also recovered the internal and external morphological characteristics of the recurrent parent, as well as its sugars and acids profile related to sensory perception. The utility of these lines was confirmed in the development of a breeding program to introgress these resistances into a `Piel de Sapo´(PS) genetic background. In this case in only two backcross generations a substantial recovery of the recurrent parent characteristics was obtained. The materials developed in this work will be very valuable for the development of melon breeding programs.

1. Introduction

Melon (*C. melo* L., *2n=2x=24*) is a valuable crop mainly cultivated in temperate and tropical areas. Its worldwide production has recently exceeded 28 Mt per year, thanks to the use of hybrid seeds and intensive cultivation systems (FAOSTAT, 2023). Nevertheless, fungal, and viral diseases constitute a serious threat to this crop, reducing both yield and fruit quality.

Cucurbits powdery mildew (CPM), mainly caused by the biotrophic fungi *Podosphaera xanthii* (*Px*) and *Golovinomyces orontii* (*Go*), can appear in both field and greenhouse conditions worldwide (Křístková *et al.*, 2009; Lebeda *et al.*, 2021). These causal agents can rapidly evolve to more virulent and better adapted genotypes that can overcome the currently used resistance genes and reduce the effectiveness of fungicides (De Miccolis *et al*., 2019). To date, the use of a set of

differential melon lines has led to the identification of more than 21 *P. xanthii* physiological races (Cui *et al.*, 2022c). Even though 239 melon accession have been described as resistant to powdery mildew, only 25 dominant resistant loci, 2 recessive loci and 7 QTLs have been mapped so far. Moreover, only a few accessions have been described as resistant to several physiological races (Cui *et al.*, 2022c).

Among the viral diseases affecting melon, those transmitted by aphids are of great importance. Different surveys recently carried out in Europe, Asia, America and Africa proved that watermelon mosaic virus (WMV) is the most prevalent virus in cucurbits fields (De Moya-Ruiz *et al.*, 2021; Desbiez *et al.*, 2020; Radouane *et al.*, 2020; Topkaya *et al.*, 2019). This *Potyvirus,* that produces chlorosis, mosaic, leaf distortion, leaf tip stunting, as well as the stop of plant growth, is transmitted in a non-persistent manner by, at least, 38 aphids species (Castle *et al*., 1992; Purcifull *et al*., 1984; Ward & Shukla, 1991). Several tolerant accessions have been described but, nowadays, only two resistant sources have been identified.

The curcurbit yellow stunting disorder virus (CYSDV) is transmitted in a semipersistent manner by *Bemisia tabaci* (Célix *et al.*, 1996). This *Crinivirus* caused severe problems in Europe during the early 2000s (Berdiales *et al.*, 1999), and, nowadays, it is devastating the cucurbits production in the United States, Mexico, and Central America (Lecoq & Desbiez, 2012; Tamang *et al.*, 2021). As other crinivirus, CYSDV produces spotted/mottled symptoms followed by extensive interveinal chlorosis. As in the case of WMV, only two resistance sources against CYSDV have been studied.

To date, the use of fungicides and insecticides has been the main strategy to limit the incidence of both fungal and vector-borne viral diseases. Nevertheless, the ability of vectors and fungi to evolve and develop resistance against these products, as well as environmental concerns, has led to the search of new strategies not based on chemicals. The introgression of genetic resistances against these pathogens from wild melon accessions into commercial cultivars is the most durable, effective, and environmentally friendly strategy to fight these pathogens.

The African accession TGR-1551 (*C. melo,* acidulus group) is resistant to *Px.* (races 1, 2 and 5), as well as to WMV and CYSDV. Moreover, the genomic regions governing these resistances have recently been narrowed, and molecular markers tightly linked to those regions have been developed. The resistance against CPM is controlled by a dominant-recessive epistasis where the dominant gene was mapped to a 250 kb region on chromosome 5 and the recessive gene was in a 381kb fragment on chromosome 12 (López-Martín *et al.*, 2022). The recessive gene that

confers resistance against WMV was narrowed to a 170 kb region in chromosome 11 and a minor QTL with modifier effects was mapped to chromosome 5 (Pérez-de-Castro *et al.*, 2019). Finally, a dominant gene that mapped to a 700 kb region in chromosome 5 confers resistance against CYSDV (Pérez-de-Castro *et al*., 2020). The dominant resistances against *Px* and CYDV have been mapped to the same region (López-Martín *et al.*, 2022). Moreover, TGR-1551 is also resistant to *A. gossypii*, as it carries the *vat* gene (Sarria-Villada *et al.*, 2009) , and it is also tolerant to *B. tabaci* (Soria *et al.*, 1999a).

The development of molecular markers, tightly linked to these genetic resistances, as well as the cost reduction in new generation sequencing technologies, have paved the way for the enhancement of breeding programs focusedin the introgression of these traits into commercial melon backgrounds. In this sense, the ibericus melon group includes commercial varieties highly appreciated by consumers. Nevertheless, to date, there are not commercial varieties resistant to WMV or CYSDV, and only a few of them carry resistances to several *Px* races. Recently, the pre-breeding line 'Carmen', resistant to CYSDV, CPM and *A. gossypii* was developed, but it did not carry the resistance gene to WMV (Palomares-Rius *et al.*, 2018).

The availability of molecular markers developed by the research group linked to the resistance regions derived from TGR-1551 has allowed the implementation of a breeding program aimed to introgress the described resistances into commercial varieties. In this work, we have monitored the effects of the introgressions from TGR-1551 and the advancement of backcrossing programs on agronomic fruit traits, and the accumulation of sugars and acids related to fruit quality, as well as the performance of the materials against WMV, CYSDV and CPM in melons of the ibericus group. New Yellow Canary (ibericus group) melon pre-breeding lines were developed, while partial advances of introgresssions in `Piel de Sapo´ (PS) pre-breeding lines are described.

2. Results and discussion

2.1. Development of the breeding program

2.1.1. Yellow type melon breeding program

The breeding program for the introgression of the resistance to WMV, CYSDV and *Px* derived from the African accession TGR-1551 into the yellow type commercial cultivar `Bola de Oro´ has been carried out by 5 backcrosses followed by 2 self-pollination cycles (**Figure 1**). To speed the breeding program, marker assisted

selection of the resistance regions was carried out since BC_3 generation and several genomic selections facilitated the recovery of the BO background.

The initial genotyping of the 200 plants that composed the BC_3 population with different sets of SNPs markers covering the genomic regions linked to the resistances to WMV, CYSDV and *Px* allowed the initial selection of 52 BC_3 plants. These plants carried different combinations of the resistance regions and were further genotyped with a set of markers that covered the whole genome (López-Martín *et al*., 2022; Pérez-de-Castro *et al*., 2019, 2020) (**Supplementary Table 1**). The 52 BC_3 genotyped plants, on average, had recovered an 83% of the BO genome (ranging from 69.83-94.82%) with an average of 6.15 introgressions/plant. The genotyping information allowed the selection of 4 BC_3 plants, which carried the resistance regions and maximized the percentage of genome recovery of the recurrent parent. This 4 selected BC_3 plants (BC_3(15), BC_3(45), BC_3(64) and BC_3(159)) had on average 7 introgression/plant and 77.66% of the BO background genome (ranging from 72.95-82.78%). The lower amount of BO genomic background in the selected plants was explained by the fact that the fine mapping of the regions associated to resistance to CYSDV and *Px* (López-Martín *et al.*, 2022; Pérez-de-Castro *et al.,* 2020) was done later than the selection. So, it was prioritized to cover a wider region of the chromosome 5 to avoid the loss of these two resistances. The percentage of BO genomic background recovered is comparable to those obtained in other BC_3 offspring used to create different melon ILs populations. For the construction of some of these mapping populations, BC_2 and BC_3 generations were also genotyped with the same set of 124 SNPs markers (Castro *et al*., 2019; Perpiñá *et al*., 2016) or with other similar approaches also based on medium-throughput SNP genotyping (Campos *et al.*, 2023; Pereira *et al.*, 2021; Santo Domingo *et al.*, 2022) . In the aforementioned studies, the average recovery of the recurrent parent genome ranged between 71.9% to 93.2% with an average of 2.6-6 introgressions/plant. In these cases, the greater recovery of the recurrent parent background genome is explained by the genomic selection carried out with the BC_2 generations, along with the selection of a unique fragment of the donor parental genome per plant.

The BC_4 offspring of the selected BC_3 plants was genotyped with the 6 HRM markers linked to the different resistance regions derived from TGR-1551 (*cysdv14, cysdv63, cysdv65, b5wmv11, b11wmv10* and *b11wmv11*) (**Supplementary Table 2**), but no background genomic selection was carried out. Since the fruits of the BC_4(159) offspring were more similar to those of BO, attending to fruit morphology and quality traits, only this family was selected to continue the breeding program. Three

BC_4 plants that carried the resistance regions (BC_4(159)-4, BC_4(159)-8 and BC_4(159)-11) were selected to obtain the BC_5 generation. All the plants of the 3 selected BC_5 families were genotyped with the HRM markers linked to the different resistance regions. Four plants were selected (BC_5(159)-4-8, BC_5(159)-4-14, BC_5(159)-8-14 and BC_5(159)-11-2). These four plants were further genotyped with a new plex of 160 SNPs markers distributed throughout the genome (**Supplementary Table 1**). A greater recovery of the BO genome was observed. These genotyped plants had an average of 5.5 introgressions/plant (range between 4-6) and 84.24% of the BO background genome (ranging from 80.82-87.67%). The BC_5(159)-8 offspring was then discarded for breeding purposes since it carried the larger number of introgressions (6).

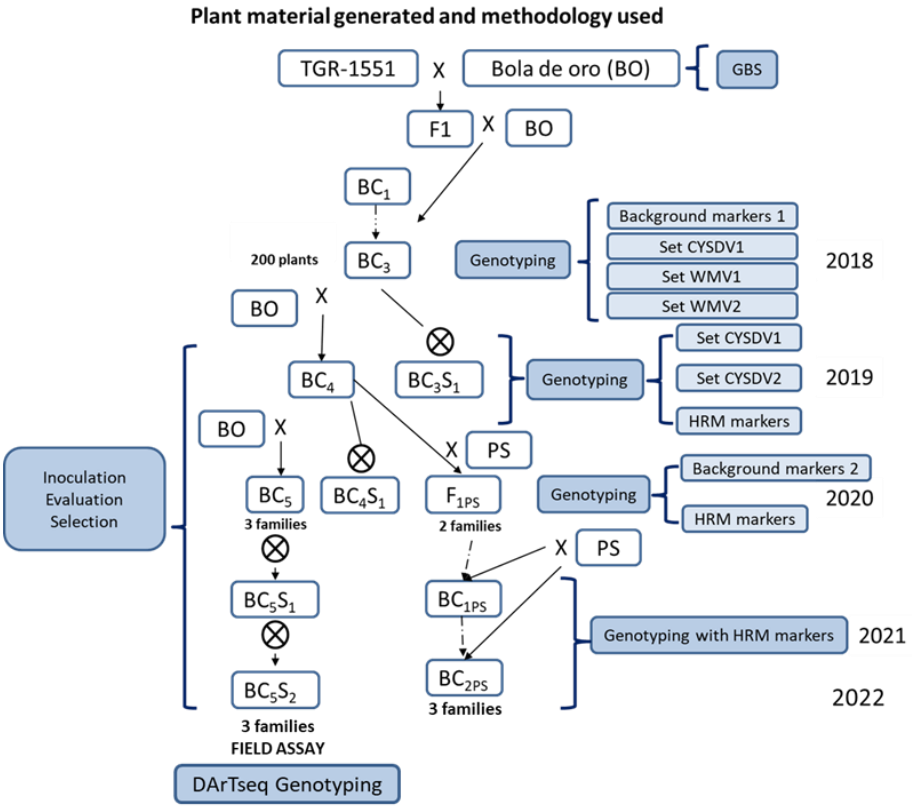


Figure 1. Breeding program for the introgression of the resistances to watermelon mosaic virus, cucurbits yellow stunting disorder virus and powdery mildew in both yellow and `Piel de Sapo´ melon types. BO:`Bola de Oro´; PS: `Piel de Sapo´

The three remaining plants, BC_5(159)-4-8, BC_5(159)-4-14 and BC_5(159)-11-2, TGR-1551 and BO, plants were then genotyped by GBS, obtaining 6,500 high quality polymorphic SNPs. With this high-density genotyping data, the average recovery of the BO background was 90.78% (ranging from 90.03-91.86%). The corresponding BC_5S_1 offspring obtained from these 3 genotyped plants were further selected with the HRM markers. Finally, those BC_5S_1 plants that produced fruits more similar to those of BO were selected to obtain the final BC_5S_2 populations (BC_5(159)-4-8-33S_2, BC_5(159)-11-2-18S_2 and BC_5(159)-4-14-42S_2). Hereinafter these populations will be named BC_5(159)-1S_2, BC_5(159)-2S_2 and BC_5(159)-3S_2, respectively. One plant of populations, BC_5(159)-1S_2, BC_5(159)-2S_2 along with TGR-1551 and BO, were genotyped with DartSeq, obtaining more than 3,700 polymorphic SNPs, that were evenly distributed through the genome. The percentage of BO recovered background was 92.33 and 92.93% for the BC_5(159)-1S_2 and BC_5(159)-2S_2 generations, respectively. The main introgressed regions associated to CYSDV, *Px* and WMV resistances were located on chromosome 5 (6,497,310 - 28,376,587 bp) and 11 (26,639,060 – 27,763,850 bp). The breeding line BC_5(159-2)S_2 had 2 additional introgressions on chromosomes 3 (26,362,068 – 27,052,604 bp) and 11 (6,911,032 – 10,760,454), whereas the introgression observed on chromosome 11 for the pre-breeding line BC_5(159-1)S_2 was larger (18,004,015 – 28,042,227bp). The introgressed regions were bigger than the previously described QTLs associated to the resistance because the initial selections were carried out before the QTLs were narrowed.

2.1.2. Piel de Sapo melon breeding program

The BC_4 plants BC_4(159)-4 and BC_4(159)-11 were crossed with the Spanish `Piel de sapo´ landrace BGCM-126 (PS). The corresponding F_1 offsprings were backcrossed two times to PS to obtain the BC_{1PS} and BC_{2PS} populations (**Figure 1**). All these offsprings were genotyped with the HRM markers linked to the resistances, previously used for the yellow type melon breeding program. In this case, no genomic selection was carried out, but those plants whose fruits were more similar to PS were selected to continue the breeding program (see section 3.4 Characterization and metabolomic analysis of PS fruits).

2.2. Resistance phenotyping results

The BO advanced backcrosses populations BC_3S_1, BC_4S_1 and BC_5S_1 were genotyped, in the corresponding season for each of them, with the HRM markers linked to the resistance regions and phenotyped for the resistance to WMV, CYSDV and *Px.* As expected according to the previous studies (López-Martín *et al*., 2022; Pérez-de-

Castro *et al*., 2019, 2020), correlation between genotype and phenotype was observed for those families that where homogeneously susceptible for one or more of the resistances, their corresponding BC_3, BC_4, BC_5 and BC_5S_2 generations were not selected to continue the breeding program.

The three selected BC_5S_2 populations were also phenotyped for the resistance to the three pathogens, showing the same resistant phenotype as TGR-1551, whereas BO showed clear infection symptoms (**Figure 2**). These results indicated that the molecular markers linked to the resistance regions derived from TGR-1551 developed in previous studies (López-Martín *et al*., 2022; Pérez-de-Castro *et al*., 2019, 2020) were useful for marker assisted selection in breeding programs. A similar approach had been followed to obtain the pre-breeding line `Carmen´, a yellow type melon also derived from the initial cross between TGR-1551 and BO, that incorporated resistance to CYSDV and *Px* (Palomares-Rius *et al.*, 2018). In the breeding program for the development of `Carmen' , selection was carried out for resistance to CYSDV; however, since *Px* and CYSDV resistances have been mapped in the same genomic region (López-Martín *et al.*, 2022), `Carmen' was also resistant to the fungus. The lines here developed, which also incorporate resistance to WMV, imply great advantage, due to the prevalence of this potyvirus in all the major producing areas worldwide.

Additionally, as TGR-1551, along with the accessions PI 414723 and IC 274006, had previously been identified as a resistance sources to Moroccan watermelon mosaic virus (MWMV) (data not shown), these BC_5S_2 families were also inoculated with this potyvirus. Nevertheless, none of the pre-breeding lines was homogeneously resistant or susceptible, which indicated that the introgressed regions involved in WMV, CYSDV and *Px* resistance did not confer resistance to MWMV. This different behavior had previously been observed when the eiF4E transcription factor was silenced in melon and it conferred resistance against MWMV but not to WMV (Rodríguez-Hernández *et al.*, 2012), indicating a possible different resistance mechanism against these two potyviruses. In any case, further studies should be carried out to better identify the resistance regions to MWMV derived from TGR-1551.

As genotypic-phenotypic correlation was observed within the BO breeding program, to date, no inoculations have been carried out with the PS advanced backcrosses. Nevertheless, it is expected to confirm the resistances with the $BC_3(BC_4xPS)S_1$ offspring.

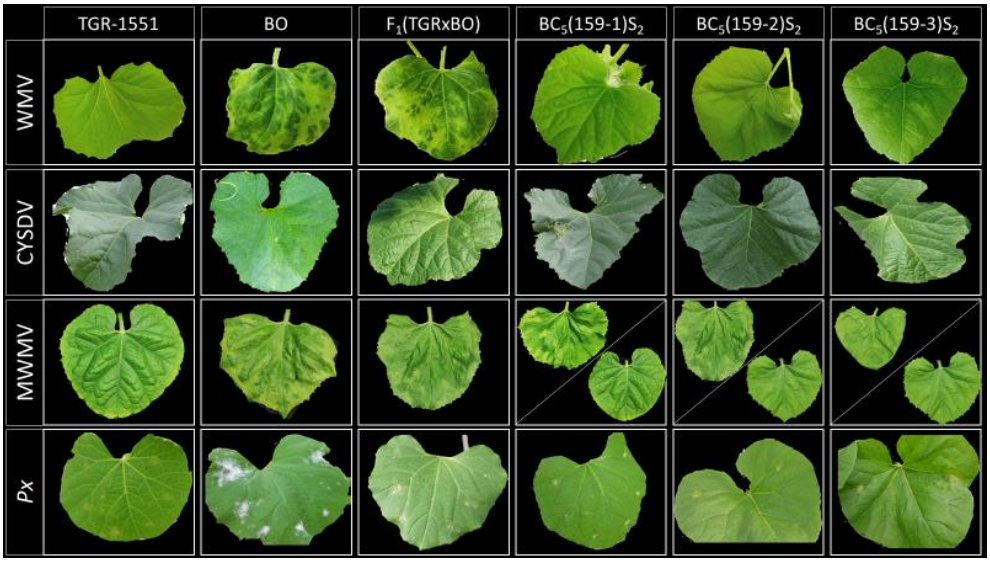


Figure 2. Phenotyping results obtained after the inoculation of the accessions TGR-1551, "Bola de oro" (BO), their F_1(TGRxBO) progeny and the 3 obtained BC_5S_2 offspring, with Watermelon mosaic virus (WMV), Cucurbits yellows stunting disorder virus (CYSDV), Moroccan watermelon mosaic virus (MWMV) and Podosphaera xanthii (Px) races 1,2 and 5.

2.3 Field evaluation of the BC_5S_2 progenies.

The 3 selected BC_5S_2 pre-breeding lines, along with the pre-breeding line `Carmen´, the accessions `TGR-1551´ and BO, and their F_1(TGRxBO) progeny, were evaluated in open field conditions to compare their yields, resistance to WMV, CYSDV and *Px* in natural conditions, as well as the organoleptic quality of the obtained fruits.

BC_5(159-2)S_2 fruits (1.93 ± 0.13 kg) were heavier than those obtained from BO (1.52 ± 0.07 kg) (**Figure 3, 4**) (**Supplementary Table 3)**, while their estimated average productivity per plant was lower (6.26 and 8.41 kg/plant, respectively) (**Figure 3**). On the other hand, BC_5(159-1)S_2 plants produced fruits with a similar weight (1.55 ± 0.05 kg) but its yield was also lower (6.61 kg/plant). Finally, BC_5(159-3)S_2 fruits were heavier (1.98 ± 0.13kg) than those collected from BO, but their yields were similar (8.65 kg/plant). As for the pre-breeding line `Carmen´, it produced smaller fruits (0.9 ± 0.01 kg) (**Figure 3, 4)**, with a similar weight to that of TGR-1551 (0.84 ± 0.03 kg) and its yield per plant was the lowest (4.64 kg/plant). Conversely, F_1(TGRxBO) fruits were the biggest (2.27 ± 0.12kg), and this genotype reached the highest yield per plant (9.51 kg/plant). The small fruit weights (FWs) obtained for both BO and `Carmen´ contrast with those previously observed under green-house

conditions, with a mean FW of 1.916 ± 0.363 and 1.857 ± 0.243kg, respectively (Palomares-Rius *et al.*, 2018).

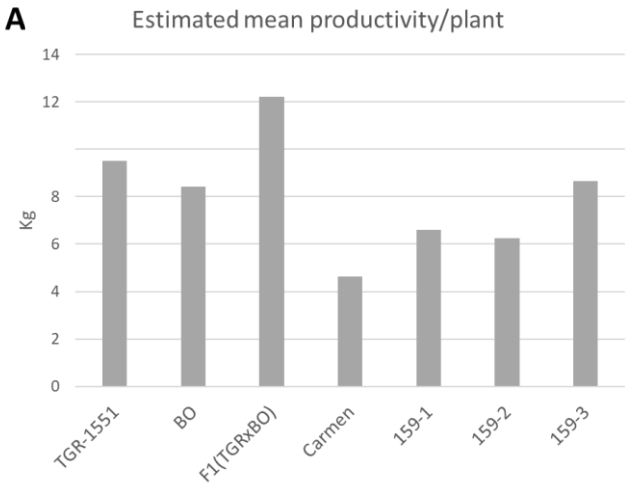


Figure 3. Estimated mean productivity obtained per plant in the field assay carried out in Comunidad Valenciana during 2022. BO: `Bola de oro´; 159-1: $BC_5(159\text{-}1)S_2$; 159-2: $BC_5(159\text{-}2)S_2$; 159-3: $BC_5(159\text{-}3)S_2$

These differences could be explained by the fact that under green-house conditions each plant only produces 1-3 fruits, which is translated into bigger fruits. However, since TGR-1551 FWs were similar to those previously reported (0.869 ± 0.161 kg) (Palomares-Rius *et al.*, 2018), the changes could also be due to the susceptibility of BO and `Carmen´ to WMV. Viral symptoms were evaluated twice during the field assay, at early- and mid-July. During both dates TGR-1551, $BC_5(159\text{-}1)S_2$, $BC_5(159\text{-}2)S_2$ and $BC_5(159\text{-}3)S_2$ plants remained asymptomatic. Whereas BO, $F_1$(TGRxBO) and `Carmen´ plants showed clear infection symptoms such as chlorosis, leaf distortion and mosaics (**Figure 5)**. Moreover, small mottled marks were also observed in the collected fruits of the susceptible varieties. Leaf tissue of the studied lines, for each independent block, was collected in mid-July and the presence of WMV, MWMV, CYSDV, CABYV and CMV was studied by PCR and RT-qPCR (**Supplementary Table 2**). Only WMV was detected in the susceptible plants of BO, F_1(TGRxBO) and `Carmen´. Surprisingly, WMV was also quantified in the first block of the asymptomatic offspring $BC_5(159\text{-}1)S_2$ (**Supplementary Figure 1)**. Partial sequences of the NIb-CP and P3-CI regions of these isolates were obtained. For the NIb-CP region, the isolate infecting the sample BO-1 (`Bola de oro´ block 1) presented a 99.77 % similarity with isolates previously detected infecting melon in Castilla la Mancha, Murcia and Alicante (Rabadán *et al.*, 2023) and a 99.07 % similarity with

the isolate EM190275. Attending the phylogenetic combination of Nib-CP and P3-CI regions as described by Desbiez *et al.* (2020), the isolate EM190275 had been classified within `Profile D' (see chapter 1) and had been observed in the same field during 2019 and 2020. As for the rest of the detected isolates, they presented a 100 % similarity with the isolate EM190221, which had been classified within `Profile A', and had also been detected in this field in previous studies. Attending the P3-CI partial sequences, the isolate infecting the sample BO-1 presented a 99.53 % similarity with isolates collected in Almería (Rabadán *et al*., 2023) and 98.83% similarity with EM190275. The other isolates were 99.52 % similar to the isolate EM190221. The availability of some `emerging' WMV isolates to overcome the resistance derived from TGR-1551 has previously been reported (Desbiez *et al*., 2021). Nevertheless, this is not the case with the detected isolates that are highly similar to isolates belonging to the EM1 group, previously reported in France (Desbiez etal., 2009) and that had been described as predominant in Comunidad Valenciana (Spain) (see chapter 1).

TGR-1551 had also been reported as resistant to CABYV, with the resistance conferred by one dominant gene and, at least, two modifier genes (Kassem *et al.*, 2015). Nevertheless, to date, no studies have been carried out to map this resistance. Even though CABYV has been present in Spain since 2003 (Juarez *et al.*, 2004) and it has recently been described as persistent in Comunidad Valenciana (see chapter 1), it was not detected in the collected samples. Regarding *Px*, it was not detected in the studied field, nor infecting melon, squash or watermelon plants.

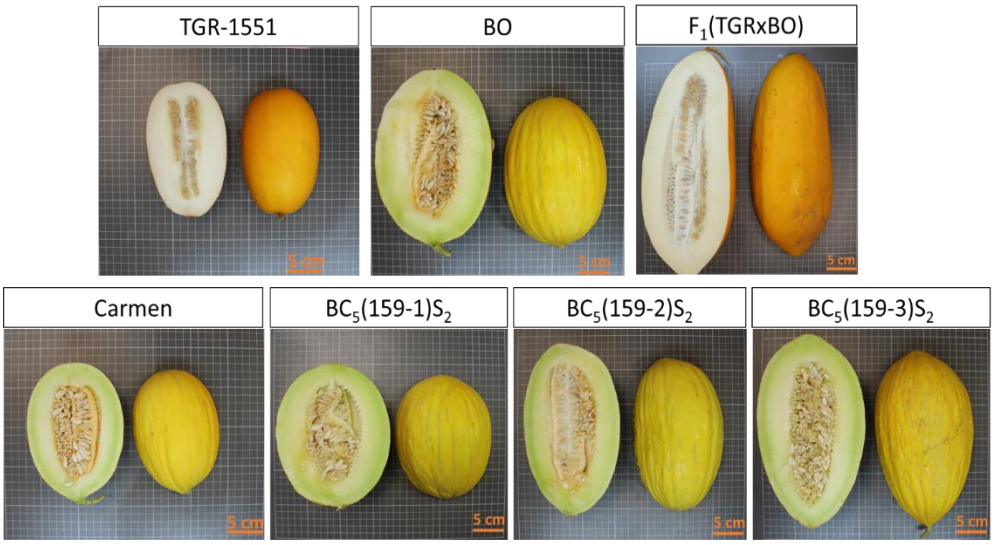


Figure 4. Full mature fruits of TGR-1551, `Bola de oro´ (BO), their $F_1$ offspring ($F_1$(TGRxBO)), the pre-breeding line `Carmen´ and the 3 pre-breeding lines obtained in this work: BC_5(159-1)S_2, BC_5(159-2)S_2 and BC_5(159-3)S_2.

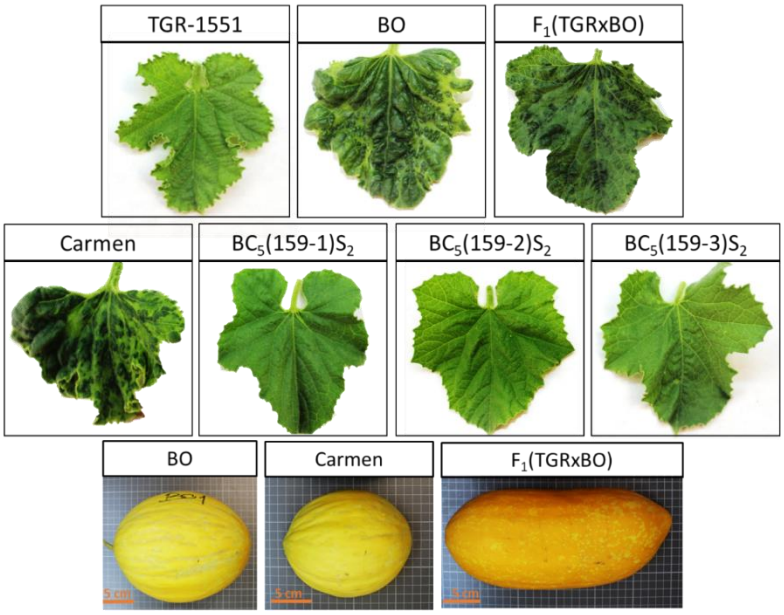


Figure 5. Viral infection symptoms observed under natural field conditions for the accessions TGR-1551, `Bola de oro´ (BO), their progeny $F_1$(TGRxBO), the pre-breeding line `Carmen´ and the 3 pre-breeding lines obtained in this work: BC_5(159-1)S_2, BC_5(159-2)S_2 and BC_5(159-3)S_2. Fruits of the susceptible lines showing chlorosis symptoms (BO, Carmen and the F_1(TGRxBO) offspring) are represented.

2.4. Characterization and metabolomic analysis of Yellow Canary fruits.

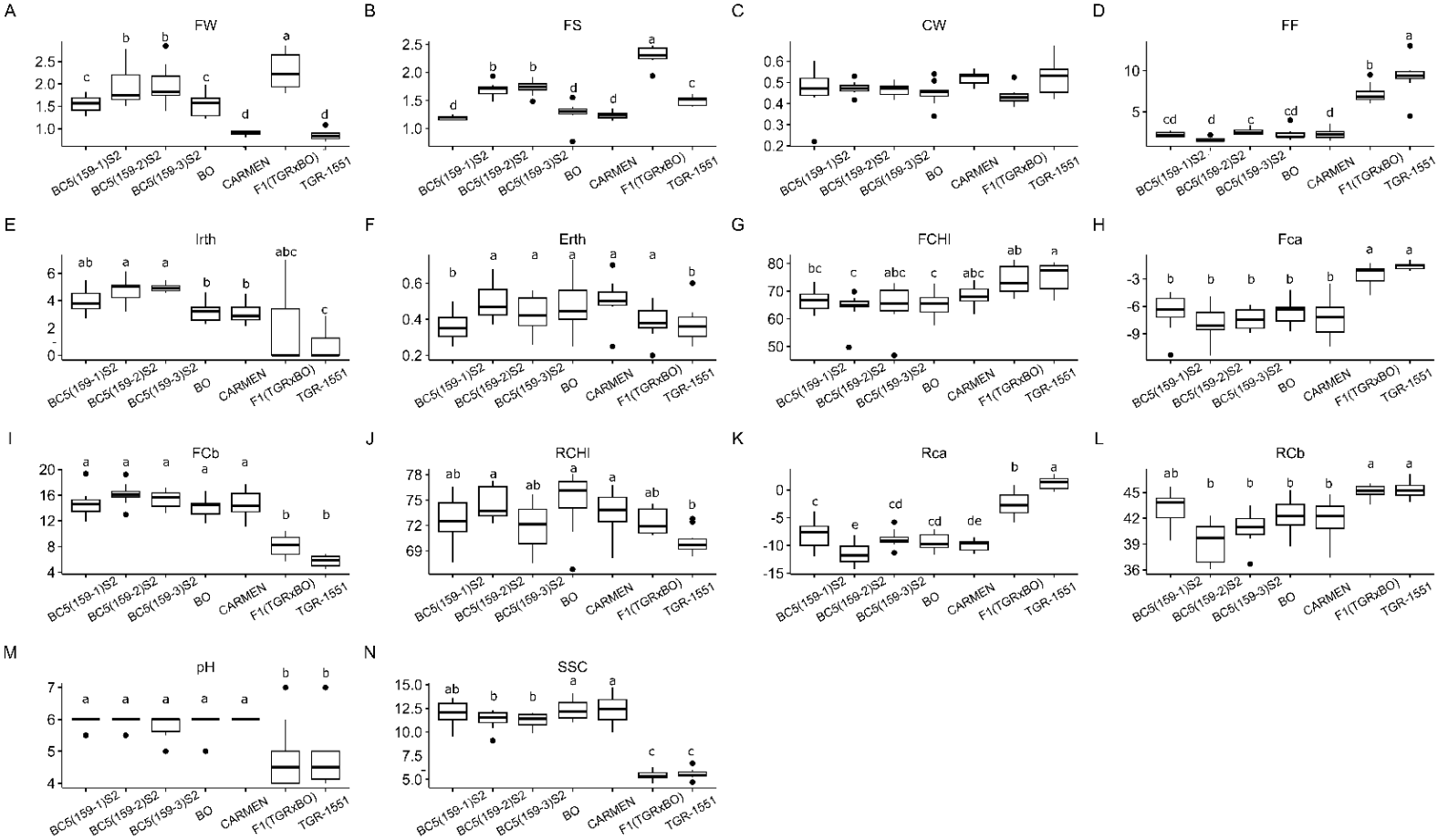
The collected fruits were characterized for traits related to fruit size and shape (FW, fruit length (FL), fruit diameter (FD) and fruits shape (FS)) (**Figure 6 A-B**) (**Supplementary Table 3**). As previously stated, only $BC_5(159\text{-}1)S_2$ fruits presented a similar weight to those of BO. This character was positively correlated with FL and FD, but not with FS, which had previously been observed in other populations (Castro *et al*., 2019; Perpiñá *et al.*, 2016). Both $BC_5(159\text{-}1)S_2$ and `Carmen´ produced rounder fruits, similar to those obtained from BO, but `Carmen´ fruits were significative smaller. As for the $BC_5(159\text{-}2)S_2$ and $BC_5(159\text{-}3)S_2$ fruits, they were more elongated and heavier (**Figure 3, 4, 6A-B) (Supplementary Table 3)**. FL, FD and FS parameters have been widely studied and numerous QTLs related to these characters have been described, most of them localizing in chromosomes 1, 2, 5, 6, 7, 8 11, 12 (Pan *et al.*, 2020). On the other hand, QTLs associated to FW have been located on all chromosomes, but only those QTLs located on chromosomes 2, 3, 8 and 11 have been identified in more than one population (Monforte *et al.*, 2014). The resistance region to WMV linked to chromosome 11 is located within the boundaries of the QTLs associated to FL, FS and FW in this region (Monforte *et al*., 2014; Perpiñá *et al*., 2016), which could explain the elongated fruits obtained from the $BC_5(159\text{-}2)S_2$ and $BC_5(159\text{-}3)S_2$, as well as the weight differences with respect to BO lines (**Figure 3, 6A-B**) (**Supplementary table 3**). Since both $BC_5(159\text{-}1)S_2$ and $BC_5(159\text{-}2)S_2$ pre-breeding lines shared a common introgression on chromosome 11 (26,639,060 – 27,763,850bp) and only the line $BC_5(159\text{-}2)S_2$ had bigger fruits (greater FW and FS) this introgressed region could not be responsible of the observed changes. However, $BC_5(159\text{-}2)S_2$ had two additional introgressions, the first of them was also located on chromosome 11 (6,911,032– 10,760,454) and the second on chromosome 3 (26,362,068 – 27,052,604bp). Since QTLs and META-QTLs have been associated to both regions (Monforte *et al.*, 2014), the observed differences could be due to these introgressions.

Attending the cavity diameter (CW) and flesh firmness (FF) no significant differences were observed between BO, `Carmen´ and the studied BC_5S_2 fruits, whereas TGR-1551 and F_1(TGRxBO) melons had a smaller cavity and harder flesh (**Figure 6 C-D**) (**Supplementary Table 3**). FC has been studied in a small number of works, nevertheless, QTLs associated to this parameter have been reported on chromosomes 1 and 2 (Castro *et al*., 2019; Paris *et al*., 2008; Perpiñá *et al*., 2016). On the other hand, QTLs located on chromosomes 1, 2, 3, 6, 7, 8 and 10 have been related to an increase in FF (Moreno *et al*., 2008; Perpiñá *et al*., 2016). The pre-breeding line $BC_5(159\text{-}2)S_2$ had a TGR-1551 introgression on chromosome 3, but

even though the introgressed region was within the boundaries of the QTL *ff3.5* described by Moreno *et al*., (2008), not significant differences with BO were observed for FF.

$BC_5(159\text{-}2)S_2$ and $BC_5(159\text{-}3)S_2$ fruits showed a thickened internal rind diameter (IRth), whereas no statistical differences were observed between $BC_5(159\text{-}1)S_2$, BO and `Carmen´ fruits (**Figure 6E**) (**Supplementary Table 3**). As for the external rind diameter (ERth) (**Figure 6F**), only those fruits obtained from the $BC_5(159\text{-}1)S_2$ plants presented a smaller diameter, equal to the one observed for TGR-1551 and F_1(TGRxBO) fruits. Rind characteristics, such as IRth, can be associated to shelf life, storage and shipping. Thus, a thickened rind could be a desirable trait in breeding programs. QTLs related to this character have been mapped on chromosomes 2, 3, 4, 6 and 7 (Castro *et al*., 2019; Perpiñá *et al*., 2016). None of the pre-breeding lines had a TGR-1551 introgression within the candidate intervals of the previously described QTLs for this parameter.

No significant differences were observed between the Hunter parameters of the flesh for the pre-breeding lines and BO (**Figure 6 G-I**) (**Supplementary Table 3**). Concerning the rind color, only the pre-breeding line $BC_5(159\text{-}2)S_2$ was different to BO for the rind color parameter a (Rca), presenting lower `a´ levels (**Figure 6 K**). The `a´ axis is relative to the green-magenta opponent colors, with lower values toward green and higher values toward magenta. However, all the developed lines had recovered the yellow rind color that is characteristic from BO and the greenish tinge detected for $BC_5(159\text{-}2)S_2$ was not really perceptible (**Figure 4**). The rind color trait had been mapped to chromosome 10 (Monforte *et al*., 2014; Perpiñá *et al*., 2016) and a Kelch domain-containing F-box protein (*CmKFB,* MELO3C011980) had also been proposed as a causal factor of the yellow rind phenotype (Feder *et al*., 2015).


A
FW
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Irth
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Erth
G
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RCHI
K
Rca
L
RCb
M
pH
N
SSC
BC5(159-1)S2
BC5(159-2)S2
BC5(159-3)S2
BO
CARMEN
F1(TGRxBO)
TGR-1551

Figure 6.

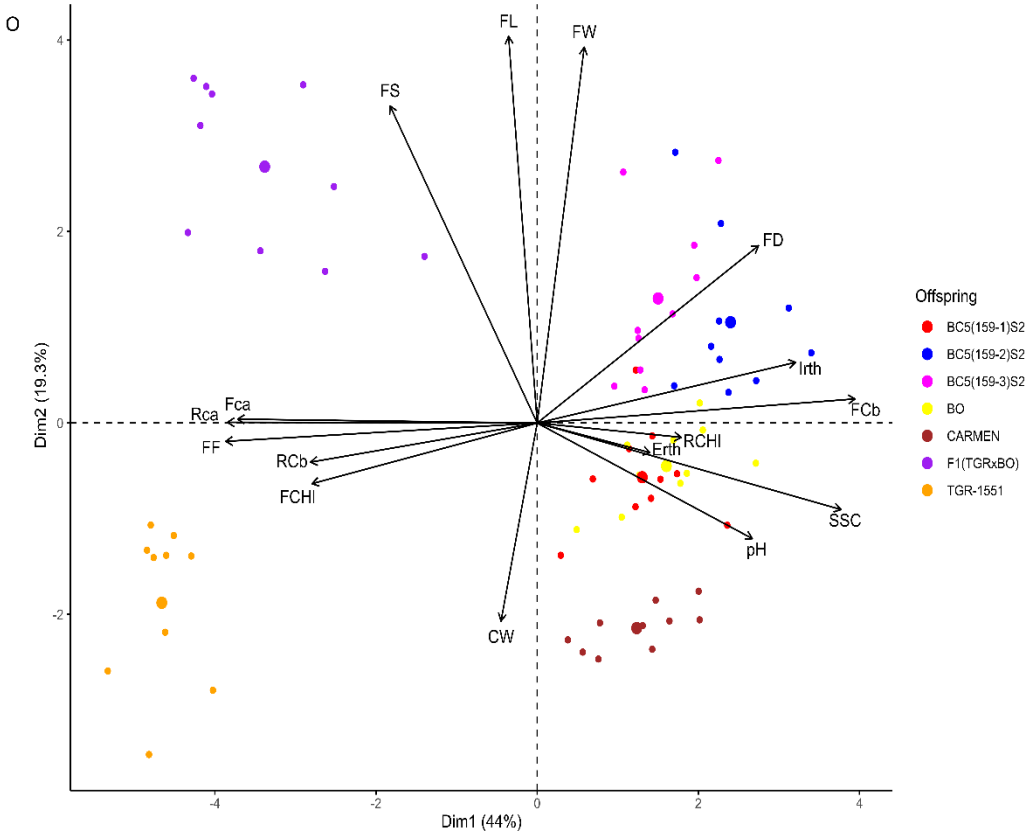

Figure 6 continuation. Comparison of the means of the morphological traits of the BC_5S_2 fruits with the mean of the recurrent parent (BO), the donor parent (TGR-1551), their F_1(TGRxBO) offspring and the pre-breeding line `Carmen´. Different letters show significantly different (p-value <0.05, LSD test). The central line within the box represents the median expression value. The box encompasses the interquartile range (IQR). The whiskers extending from the box indicate the minimum and maximum values within 1.5 times the IQR. Data points beyond the whiskers are represented as individual points and are considered outliers. Different letters indicate statistical differences (LSD test, p-value < 0.05) (A-H). Principal component analysis (PCA) or organoleptic traits. The bigger points indicate the average value of the corresponding genotype or pre-breeding line (O). The evaluated traits were: FW: fruit weight in kg; FL: fruit length in cm; FD: fruit diameter in cm, FS: fruit shape as the ratio between fruit length and fruit diameter; CW = cavity width in cm; FF: flesh firmness in kg/cm2; Irth: internal rind thickness in mm; Erth: external rind thickness in mm; Hunter coordinates, FCHI = flesh color luminosity, FCa = flesh color a parameter, FCb = flesh color b parameter; RCHI = rind color luminosity, RCa = rind color a parameter, FCb = rind color b parameter; pH; SSC: soluble solids content in Brix degree.

As a summary, the PCA plot (**Figure 6 O**) showed that BC_5(159-1)S_2 fruits had recovered the external and internal characteristics of BO. TGR-1551 and

F1(TGRxBO) fruits were separated from the rest of the studied fruits in the X axis due to their orange rind color and the white flesh color. However, they formed two different clusters in the Y axis due to the differences in FS and FW, since F_1(TGRxBO) fruits were the heaviest and largest. BC_5(159-1)S_2 fruits were more similar to BO and they clustered together in the PCA analysis. The main differences between the other two pre-breeding lines and BO were observed in the Y axis, since their fruits wee larger, heavier and had a thickened internal rind diameter. Conversely, `Carmen´ fruits formed a different cluster due to their smaller size and weight and also because of its larger CW.

Acidity, sugars and flavor volatiles are the major determinants of the taste and quality of most fruits. Cultivated sweet dessert melons usually have low levels of acidity, whereas their wild-type relatives, such as TGR-1551, have acidic fruits when mature. `Carmen´, as well as the BC_5S_2 lines had low levels of acidity, with pH values of 6, the same as BO (**Figure 6M) (Supplementary Table 3)**. This character is controlled by a single dominant *locus* named *PH* (MELO3C025264), located on chromosome 8 (Cohen *et al.*, 2014).

As for the soluble solid content (SSC), all the pre-breeding lines showed significative differences with TGR-1551 but only `Carmen´ and BC_5(159-1)S_2 fruits showed no significative differences with BO (**Figure 6N) (Supplementary Table 3)**. The genetic control of sugar accumulation is very complex and a large number of QTLs related to this trait have been reported (Diaz *et al.*, 2011). However, only those QTLs mapped on chromosomes 2, 3 and 5 have been identified as meta-QTLs, as they have been detected in two or more independent experiments (Monforte *et al.*, 2014).

The content of specific sugars (sucrose, fructose and glucose) was also measured. Our three BC_5S_2 pre-breeding lines showed a lower concentration of SE and TS than BO and `Carmen´ (**Figure 7 A, B**) (**Supplementary Table 4**). In the case of BC_5(159-2)S_2 and BC_5(159-3)S_2 lines, these differences were due to a lower accumulation of sucrose (**Figure 7F**) and higher HS ratio (**Figure 7C**) (**Supplementary Table 4**). On the other hand, BC_5(159-1)S_2 fruits had significant lower levels of glucose, fructose and sucrose, but a HS ratio similar to BO **(Figure 7 C-F)**. This sugar profile, along with the higher concentration of citric acid, makes this line the one that shows the most different taste-related sugar and acid profile when compared to BO (**Figure 7 I**). On the other hand, despite the lower sucrose accumulation, both BC_5(159-2)S_2 and BC_5(159-3)S_2 fruits, showed a sugars and acids profile similar to BO **(Figure 7 I)**. It cannot be ruled out that the high HS ratio might be indicating a delayed ripening, resulting in lower sugar levels. Nonetheless, these lines has a similar or even lower

flesh firmness compared to BO and this possibility is unlikely. Park *et al*., (2009), using a F_2 population derived from 'Deltex' (ameri group) and TGR-1551, observed QTLs on chromosomes 2, 3, 4, 6, and 11 related to soluble solids content, sucrose, fructose, and glucose. Thereby, the different profiles observed between BC_5(159-1)S_2 and BC_5(159-2)S_2 pre-breeding lines could be due to the larger introgression on chromosome 11 (18,004,015 – 28,042,222 bp) observed in the line BC_5(159-1)S_2. Moreover, the additional introgressions on chromosomes 3 (26,362,068 – 27,052,604 bp) and 11 (6,911,032 – 10,760,454 bp) observed in the pre-breeding line BC_5(159-2)S_2 could also negatively affect the accumulation of sugars.

The PCA showed that TGR-1551 and F_1(TGRxBO) fruits formed a single cluster, separated from the rest of the melons in the X axis due to their higher ratio HS and malic acid content, as well as for the lower accumulation of sugars. A strong dominant effect in the metabolic profile was thus also confirmed. The pre-breeding line that showed the more different profile was BC_5(159-1)S_2, since it was separated from in the Y axis because of its higher concentration of citric acid and lower fructose and glucose contents. BC_5(159-2)S_2 and BC_5(159-3)S_2 were more similar to BO, however they did not cluster together due to the lower SE, TS and sucrose accumulation. `Carmen´ was more similar to BO, but it showed a higher trend to accumulate citric acid, as BC_5(159-1)S_2.

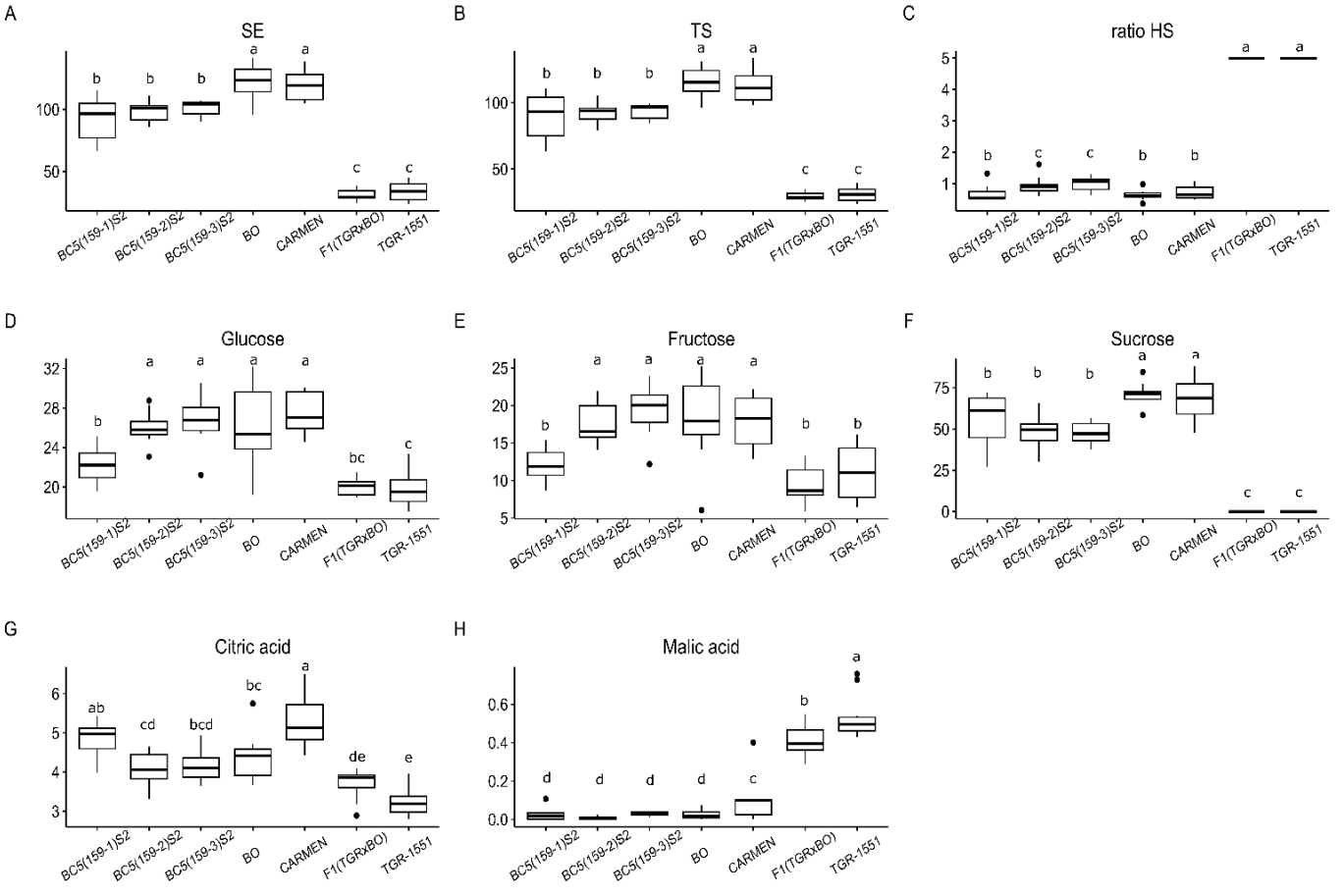
A
SE
B
TS
C
ratio HS
D
Glucose
E
Fructose
F
Sucrose
G
Citric acid
H
Malic acid
BC5(159-1)S2
BC5(159-2)S2
BC5(159-3)S2
BO
CARMEN
F1(TGRxBO)
TGR-1551

Figure 7.

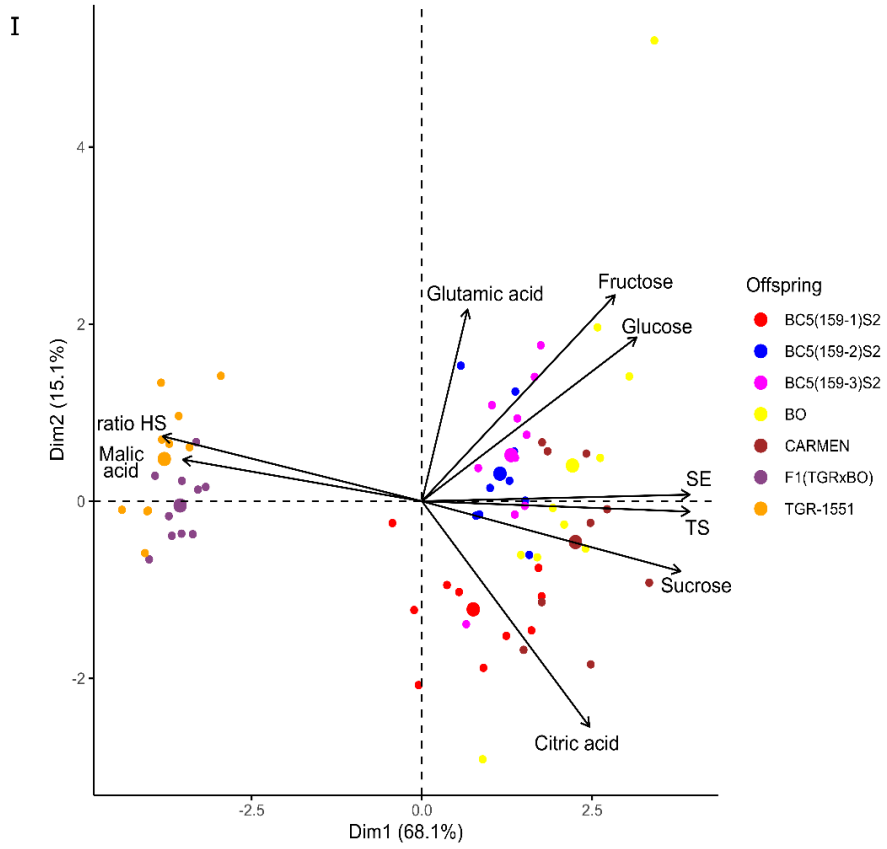

Figure 7.Continuation. Sugars and acids profile of the BC_5S_2 fruits the recurrent parent (BO), the donor parent (TGR-1551), their F_1(TGRxBO) offspring and the pre-breeding line `Carmen´. Different letters show significantly different (p-value <0.05, LSD test). The central line within the box represents the median value. The box encompasses the interquartile range (IQR). The whiskers extending from the box indicate the minimum and maximum values within 1.5 times the IQR. Data points beyond the whiskers are represented as individual points and are considered outliers (A-H). Principal component analysis (PCA) of sugars and acids accumulation related to sensory perception. The bigger points indicate the average value of the corresponding genotype or pre-breeding line (I). SE: sucrose equivalents; TS: total sugars; ratio HS: ratio hexoses-sucrose.

From the practical point of view, these results showed that the genetic background of TGR-1551 has a strong dominant effect, with a negative impact in the accumulation of organic acids and sugars. However, five backcross generations were enough to nearly restore the metabolic profile determining the sensory characteristic of the fruits. On the other hand, it also stresses the necessity to evaluate a precise determination not only of CSS but also of the accumulation of

individual sugars and acids and to perform differential selections during the development of the program. Indeed, CSS would not identify the effects on citric acid accumulation or the changes observed in the HS ratio.

2.4 Characterization and metabolomic analysis of PS fruits.

The utility of the Yellow Canary pre-breeding lines was confirmed using them to perform a rapid introgression of resistances in the PS background. Regarding this breeding program, to date, two back-crosses to the recurrent parental BGCM-126 have been carried out. However, a great advance in the recovery of the PS phenotype has already been observed. Thus, confirming the advantages of pre-breeding lines with a high level of melon genome recovered. Firstly, FW has increased, CW has been narrowed and a more elongated FS has been obtained, which are typical characteristics of PS fruits when compared to yellow type fruits (**Figure 8 A-C, 9)** (**Supplementary Table S5**). Regarding Irth and Erth, significant statistical differences between the pre-breeding lines and PS fruits were only observed during 2022 for the BC_2 fruits of some of the lines (**Figure E-F**) (**Supplementary Table 5B**). With respect to the flesh color, while BO flesh is white-green, PS tends to be whiter. This characteristic was already recovered in the F_1 generation (**Figure 8 G-I**, **9**) but some differences were observed between BC_2 and PS fruits. As for the external rind color, BO and PS show clearly contrasting phenotypes. Even though the general appearance of the BC_2 fruits is more similar to PS, an intermedium, yellowing phenotype was yet appreciated, which was reflected in the RCHI and RCb color parameters (**Figure 8 J-L, 9**).

The PCAs carried out with each generation show the advance in the breeding program (**Figure 8 N-P)**. During the 3 years of study, BO and PS fruits have been separated along the X axis in two different clusters mainly due to internal and external color parameters, as well as for the CW. F_1 fruits showed an intermedium phenotype (**Figure 8 N).** Even though some dispersion was observed within BC_1 fruits, they were already clustered with PS fruits (**Figure 8 O).** BC_2 fruits were also clearly grouped in a distinct cluster from BO fruits, however, certain differences were observed with respect to PS fruits, mainly due to flesh color and FF (**Figure 8 P**). Additional back-crosses should be carried out to recover a complete PS phenotype.

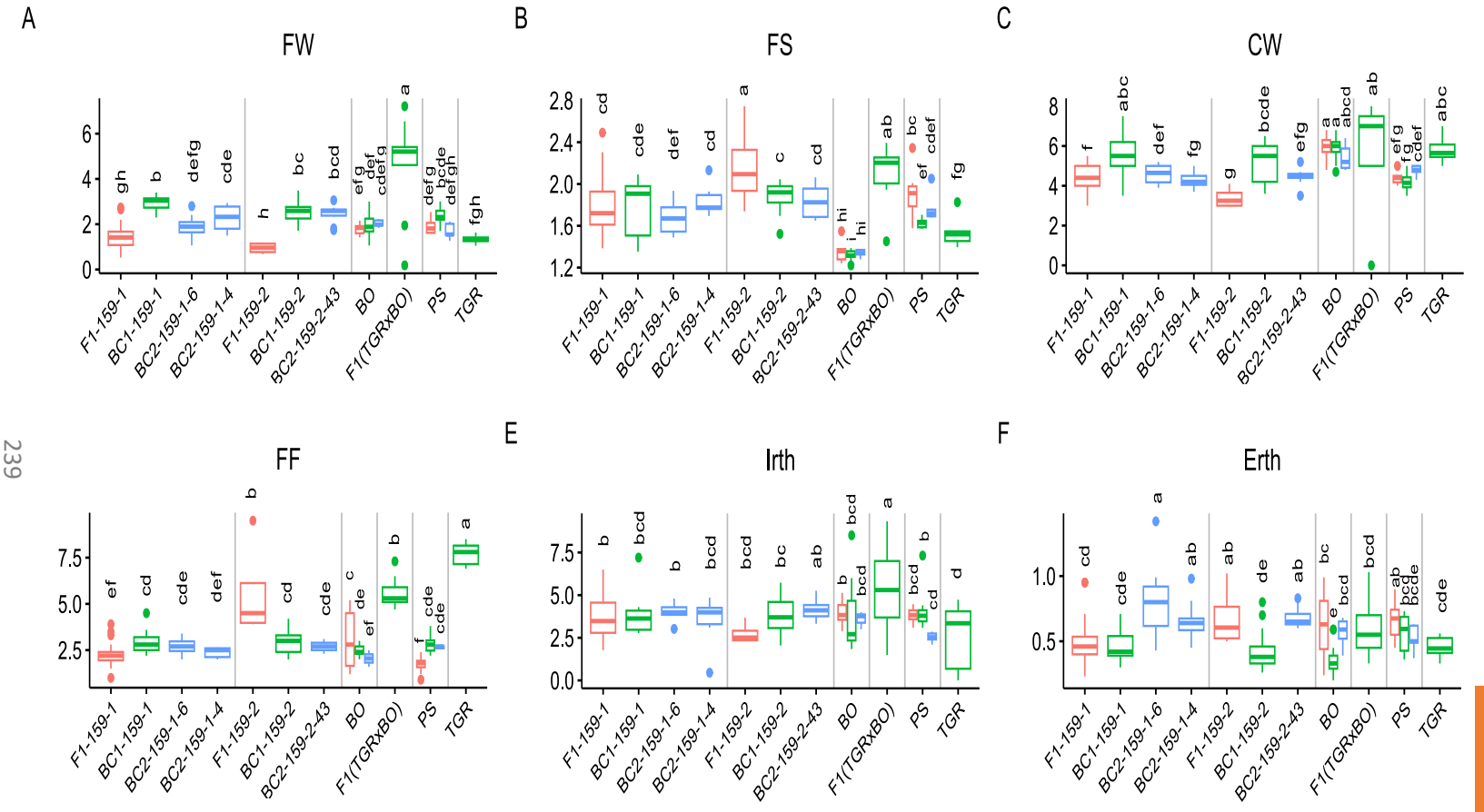
A
FW
B
FS
C
CW
E
Irth
F
Erth
F1-159-1
BC1-159-1
BC2-159-1-6
BC2-159-1-4
F1-159-2
BC1-159-2
BC2-159-2-43
BO
F1(TGRxBO)
PS
TGR

Figure 8.

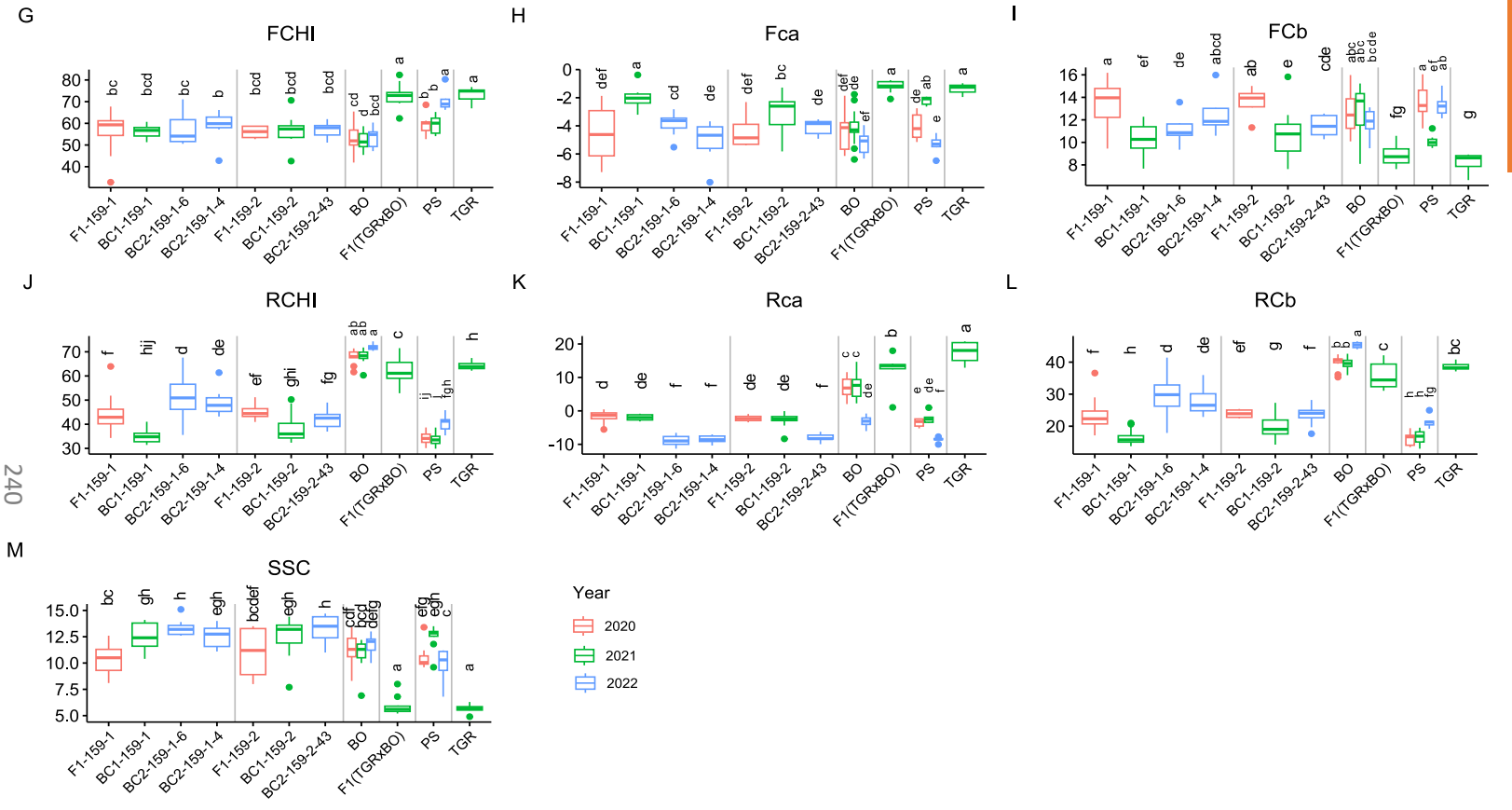

Figure 8. Continuation

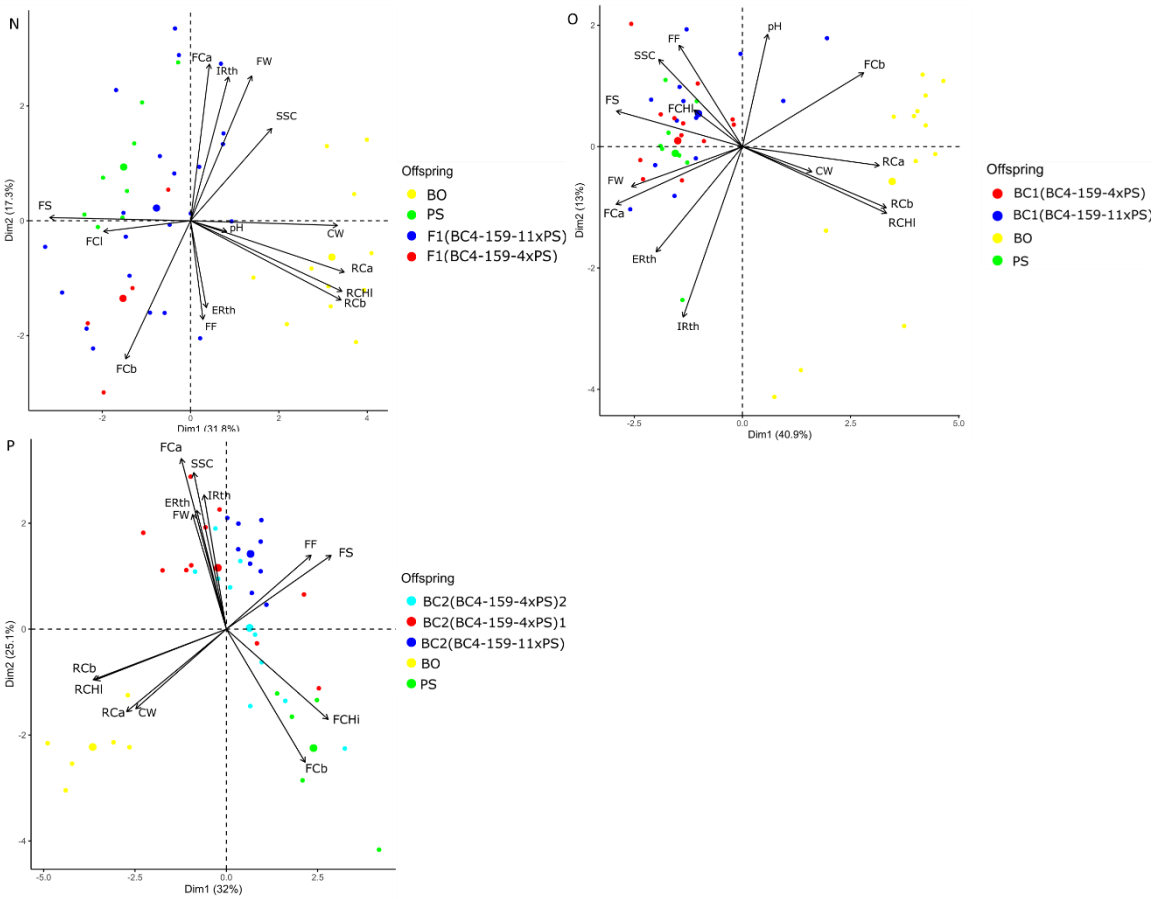

Figure 8. Continuation. Comparison of the means of the morphological traits of the F1, BC1 and BC2 fruits with the mean of the recurrent parent (PS), the donor parents (TGR-1551 and BO), and the F1(TGRxBO) offspringDifferent letters show significantly different (p-value <0.05, LSD test). The central line within the box represents the median expression value. The box encompasses the interquartile range (IQR). The whiskers extending from the box indicate the minimum and maximum values within 1.5 times the IQR. Data points beyond the whiskers are represented as individual points and are considered outliers. Different letters indicate significative differences (LSD test, p-value <0.05) (A-M). Principal component analysis (PCA), the bigger points indicate the average value of the corresponding genotype or pre-breeding line (N-P). The evaluated traits were: FW: fruit weight inkgs; FL: fruit length in cm; FD: fruit diameter in cm, FS: fruit shape as the ratio between fruit length and fruit diameter; CW = cavity width in cm; FF: flesh firmness in kg/cm^2; Irth: internal rind thickness in mm; Erth: external rind thickness in mm; Hunter coordinates, FCHl = flesh color luminosity, FCa = flesh color a parameter, FCb = flesh color b parameter; RCHl = rind color luminosity, RCa = rind color a parameter, FCb = rind color b parameter; pH; SSC: soluble solids content in Brix degree.

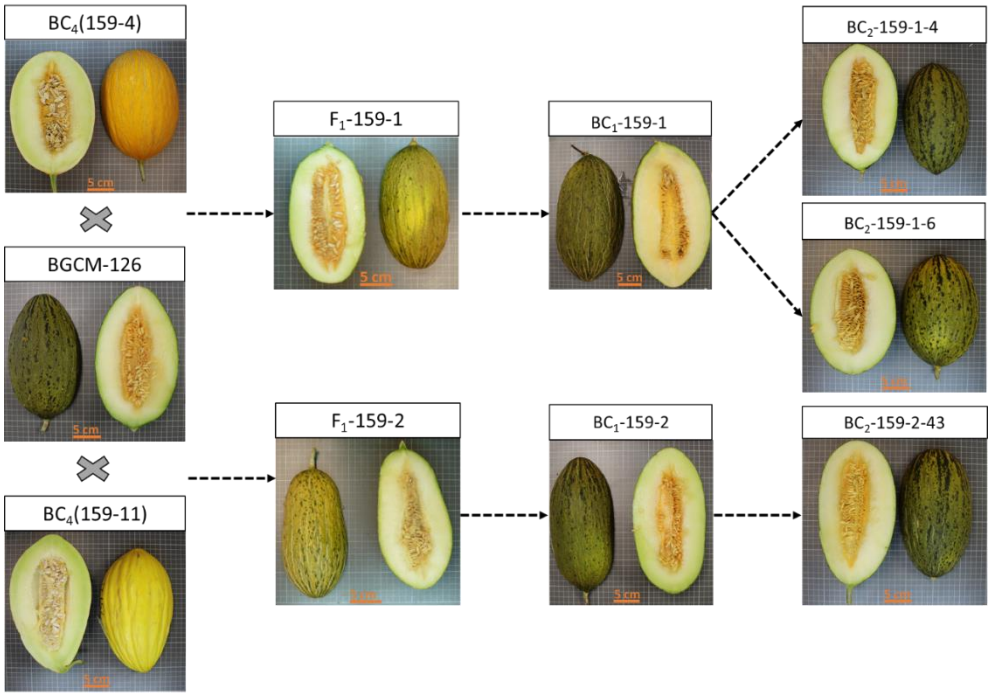

Figure 9. Full mature fruits representing the phenotypic advance in the PS breeding program thought generations.

Additionally, an increase in SSC was observed in BC generations with respect to the F_1. Regarding the sugars profile, BC_2 offspring had higher SE and TS concentrations and a lower ratio HS than PS (**Figure 10 A-C**) (**Supplementary Table 6**). In fact, the concentration of SE, TS and sucrose observed in the pre-breeding lines BC_2(159-1-6) and BC_2(159-243) was higher that the concentrations observed in both BO and PS parental lines. This potential transgressive effect observed in the breeding lines could be explained by the new allelic combinations or epistatic interactions between BO and PS genetic backgrounds. This kind of segregation has previously been observed in different melon ILs populations, affecting different quality parameters (Campos *et al*., 2023; Perpiñá *et al*., 2016; Zamir, 2001). Nonetheless, an effect accelerating ripening cannot be completely discarded, However, flesh firmness in these lines was not lower than in the PS recurrent parent, apparently discarding that the fruits had ripened earlier and accumulated more sugars in plant. Regarding the acid profile, BO accumulated more citric acid and less malic acid than PS (**Figure 10 G-H**). Whereas F_1 and BC_1 offspring showed similar concentrations to those observed for BO, BC_2 populations were more similar to PS fruits

(**Supplementary Table 6**). This result confirms a dominant effect of citric acid accumulation in BO, but it can be easily restored in alternative backgrounds.

The effect of the development of the breeding programs on the accumulation of sugars and acids conditioning the sensory profile of melon fruits could be traced in the PCAs obtained for each generation (**Figure 10 I-K**). F_1 fruits showed an intermediate phenotype between BO and PS and did not cluster with PS fruits due to their lower fructose, glucose and ratio HS (**Figure 10 I**). Among both selected F_1 populations, F_1-159-1 tended to show higher citric acid accumulation than F_1-159-2. In 2021, the conditions favored the performance of PS, which reached a high level of TS and SE similar to BO, and peaked malic acid contents. This behavior resulted in low values of HS ratio. BC_1 fruits sugar and acids profile did not overlap the recurrent parent. Although TS and SE levels were similar to those obtained in PS, the acidic profile was more similar to BO, with higher citric and lower malic acid contents (**Figure 10 J**).

BC_2 generations were grown in 2022. A high advance was achieved in this generation with a drastic reduction in citric acid contents, resembling the recurrent parent. Consequently, these generations were closer to PS in the PCA and maintained a clear distance to BO (**Figure 10 K**). Still, some differences remained between BC_2 populations and PS, as they clearly tended to accumulate higher sugar contents, leading to higher TS and SE values, a performance closer to the high sugar accumulation of BO. Nonetheless, this behavior is not detrimental to fruit quality. On the contrary it could be considered as an advance from the sensory point of view. In general the spectra of variation of the three BC_2 lines tended to overlap. Among the BC_2 lines derived from F_1-159-1, BC_2-159-1-6 tended to be less variable in the accumulation of sugars and acids

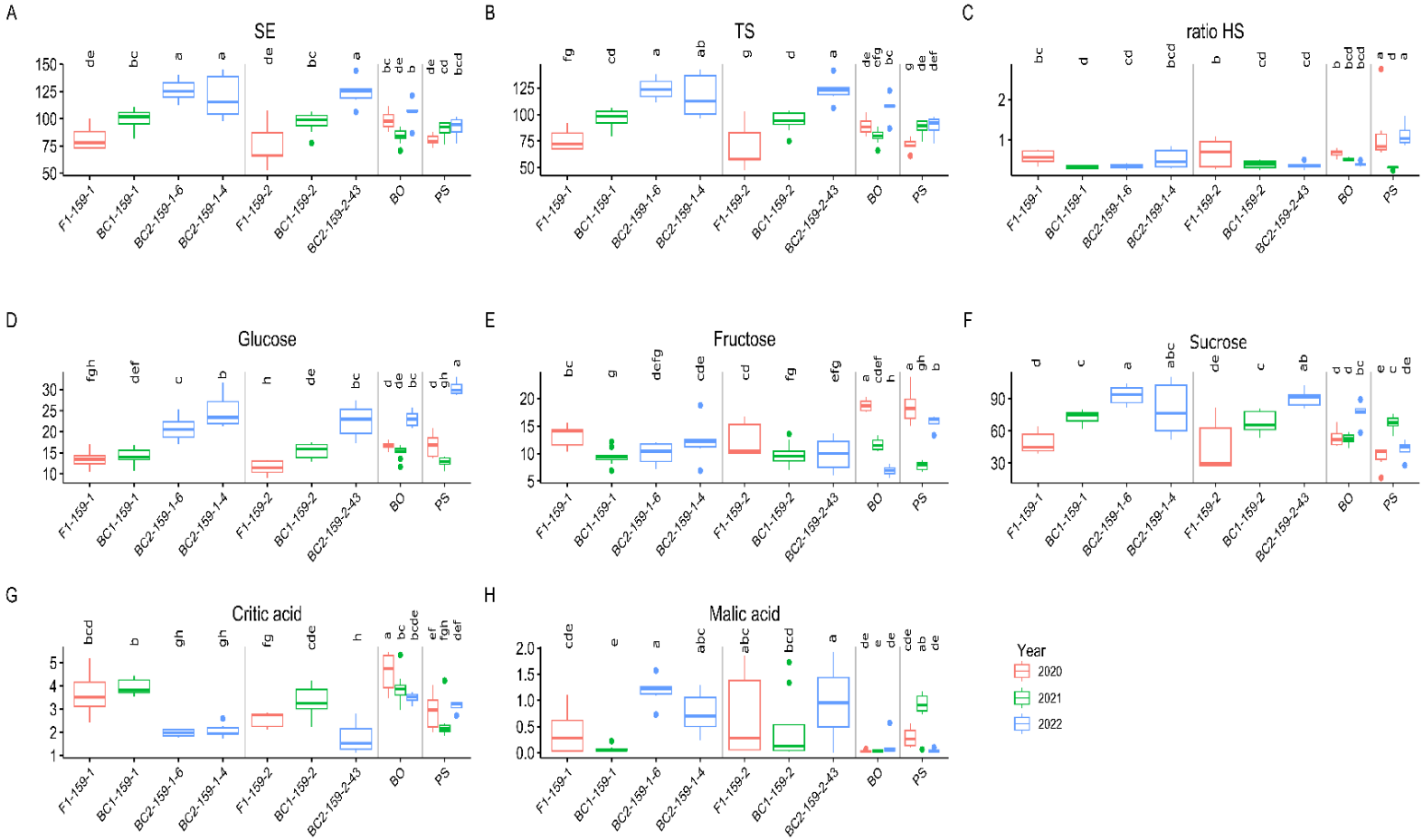
A
SE
B
TS
C
ratio HS
D
Glucose
E
Fructose
F
Sucrose
G
Critic acid
H
Malic acid
F1-159-1
BC1-159-1
BC2-159-1-6
BC2-159-1-4
F1-159-2
BC1-159-2
BC2-159-2-43
BO
PS
Year
2020
2021
2022

Figure 10.

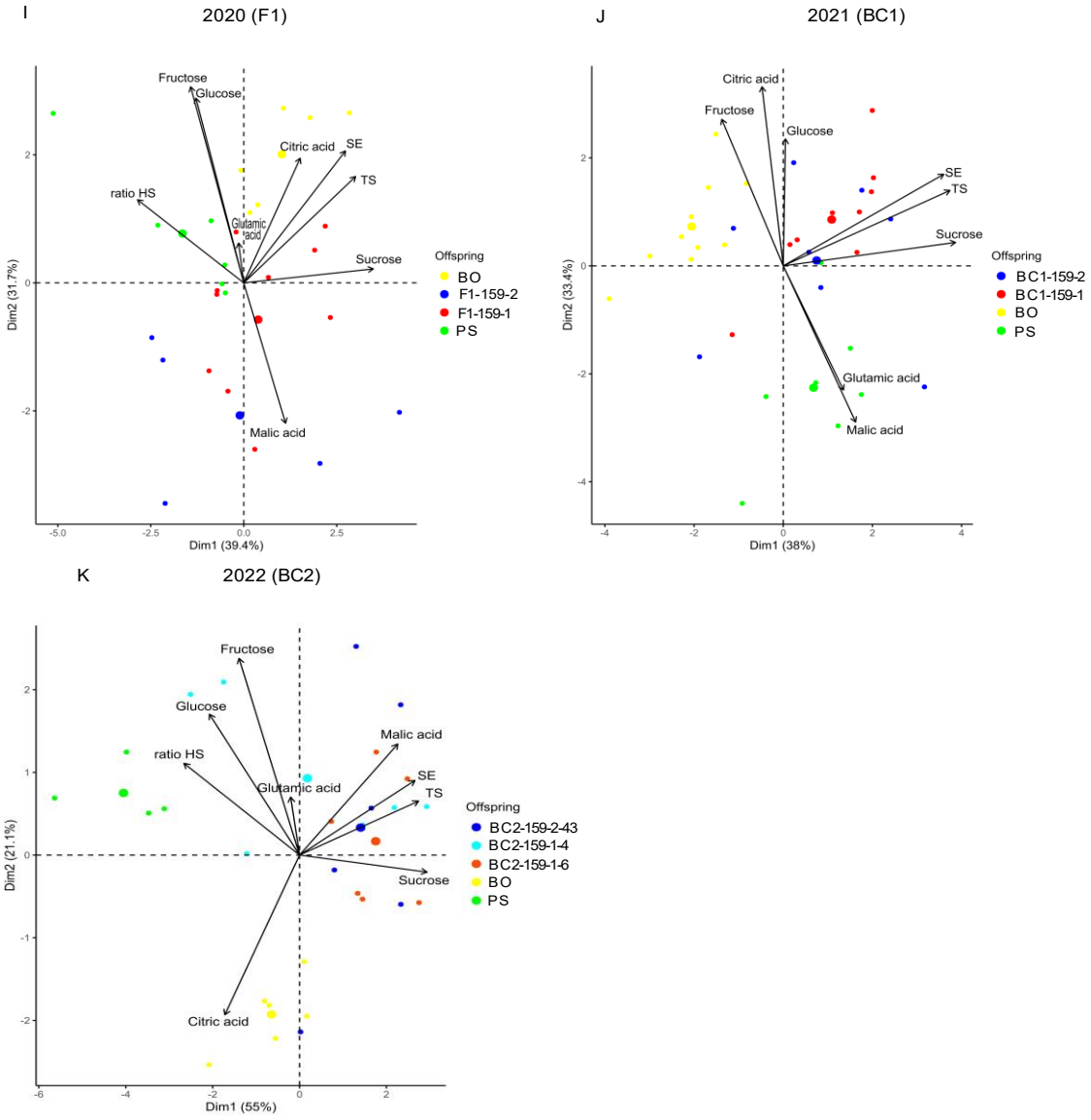

Figure 10. Continuation. Sugars and acids profile of the $F_{1PS,}$,BC_{1PS} and BC_{2PS} fruits, the recurrent parent (PS), and the donor parent (BO). Different letters show significantly different (p-value <0.05, LSD test). The central line within the box represents the median value. The box encompasses the interquartile range (IQR). The whiskers extending from the box indicate the minimum and maximum values within 1.5 times the IQR. Data points beyond the whiskers are represented as individual points and are considered outliers (A-H). Principal component analysis (PCA) of sugars and acids accumulation related to sensory perception. Bigger points indicate the average value for each genotype or pre-breeding line (I-K). SE: sucrose equivalents; TS: total sugars; ratio HS: ratio hexoses to sucrose.

3.Conclusions

The pre-breeding lines obtained in this work derived from TGR-1551 constitute a valuable resource for breeders has they have been proven to be resistant to WMV, CYSDV and *Px*. Five backcrossing generation were required to obtain a substantial recovery of both morphological and metabolomic profile of the Amarillo type melon. The utility of these materials was confirmed in the introgression of this resistances in the Piel de Sapo background. In this case only two backcrossing generations were required to recover the recurrent parent traits, even improving sugar accumulation. These materials represent a valuable tool for the development of multi-resistant elite melon breeding lines with a minimum impact in the metabolomic profile related to sensory perception.

4. Materials and methods

4.1. Plant material and breeding scheme

The Zimbabwean melon cultivar TGR-1551 (acidulus group), used as resistance source in this study, had been described as resistant to *Px* (races 1, 2 and 5), WMV and CYSDV (Pérez-de-Castro *et al.*, 2019; Pérez-De-castro *et al.*, 2020; López-Martín *et al.*, 2022). It was initially crossed to the Spanish susceptible yellow type cultivar 'Bola de oro' (BO) and further backcrossed three times to generate 200 BC_3 plants (**Figure 1**). Those plants were genotyped and 52 of them were selected for carrying the resistance regions with the lesser donor parental background. The selected plants were then backcrossed and self-pollinated to obtain the corresponding BC_4 and BC_3S_1 families, respectively. BC_4 families were also genotyped and the selected plants were backcrossed and self-pollinated to obtain the BC_5 and BC_4S_1 generations. The offspring of the three plants that produced fruits more similar to those obtained from BO were selected to continue the breeding program. The three selected BC_5 families were further genotyped, selected and self-pollinated to obtain the corresponding BC_5S_1 offspring. The better plants to continue with the program were selected as previously described. The three selected BC_5S_1 families were also genotyped and three BC_5S_2 families were obtained. During the development of the breeding program BC_3S_1, BC_4S_1, BC_5S_1 and BC_5S_2 offspring were phenotyped for the resistance to WMV, CYSDV and *Px* to confirm that the selection method was effective.

To introgress the resistances into a PS genetic background, three BC_4 plants carrying the regions associated to the resistances were crossed to the PS accession BGCM-126 (**Figure 1**). To date, three backcrosses have been carried out to obtain plants with three backcrosses to PS (BC_3 seeds). The F_{1PS} (from the cross of the selected

BC_4xPS), BC_{1PS}(F_{1PS}xPS, that is (BC_4xPS)xPS) and BC_{2PS}(BC_{1PS}xPS) families were also genotyped and selected in the same manner as it had been done in the yellow type breeding program (**Figure 1**). Plants of generations F_{1PS}, BC_{1PS} and BC_{2PS} fruits have been evaluated for quality traits.

4.2. Molecular markers and genotyping methods.

For the different genotyping selections, genomic DNA was extracted from young leaves following the Doyle & Doyle (1990) method. DNA was quantified using spectrophotometry via a Nanodrop ND-1000 Spectrophotometer v.3.5 (LabTech International, Heathfield, UK) and adjusted to a final concentration of 10 ng/µl for Sequenom and 50 ng/µl for the High-resolution melting (HRM), genotyping by sequencing (GBS) and DArTseq analysis.

Initially, BC_3 plants were genotyped with the sets of molecular markers CYSDV1, WMV1 and WMV2 (Pérez-de-Castro *et al*., 2019; Pérez-de-Castro *et al*., 2020) (**Supplementary Table 1**), implemented in the Agena Bioscience iPLEX® Gold MassARRAY platform by the Epigenetic and Genotyping unit of the University of Valencia (Unitat Central d'Investigació en Medicina (UCIM), Valencia, Spain). A total of 52 BC_3 plants were selected for carrying the different resistance regions and were further genotyped with a set of 124 molecular markers distributed throughout the genome (Esteras *et al.*, 2013; López-Martín *et al.*, 2022). Those plants with the minimum TGR-1551 background genome were selected to continue with the breeding program. BC_3S_1, BC_4, BC_4S_1, BC_5, BC_5S_1, BC_5S_2, F_{1PS}, BC_{1PS} and BC_{2PS} families were further genotyped with the HRM markers *cysdv63* and *cysdv65* (**Supplementary Table 2**), linked to the resistances to CPM and CYSDV in chromosome 5 (López-Martín *et al*., 2022; Pérez-de-Castro *et al*., 2020), the markers *cysdv14* and *cysdv17*, linked to a minor CYSDV resistance QTL in chromosome 5 (Pérez-de-Castro *et al*., 2020) and also with the newly developed HRM markers (based on markers included in the sets WMV2 of the Agena Bioscience iPLEX® Gold MassARRAY platform) *b11wmv10, b11wmv11 and b5wmv11* (**Supplementary Table 2**), which are linked to the resistance to WMV (Pérez-de-Castro *et al.*, 2019). Additionally, BC_3S_1 and BC_4 offspring were also genotyped with the plexes CYSDV1 and CYSDV2 (Pérez-de-Castro *et al*., 2020). In order to select those plants carrying less genetic background from TGR-1551, some plants from the BC_4S_1, BC_5 and BC_1(BC_4xPS) generations were genotyped with a set of 160 markers evenly distributed throughout the genome (López-Martín *et al.*, 2022) (**Supplementary Table 1**). Selected BC_5 plants, TGR-1551 and BO were also genotyped by GBS. Finally, one plant of each BC_5S_2 line, as well as BO and TGR-1551, were genotyped with DArTseq (Diversity Arrays Technology Pty Ltd,

https://www.diversityarrays.com/services/dartseq/), an alternative genotyping-by-sequencing system. Both lectures from GBS and DArTseq experiments were mapped to the reference melon genome v.4.0 (Castanera *et al.*, 2020) using bowtie2 v.2.3.4. (Langmead & Salzberg, 2012) and SNP calling was carried out with Freebayes v.1.3.4 (Garrison & Marth, 2012b).

4.3. Phenotyping for resistance to WMV, CYSDV and *Px*

BC_3S_1, BC_4S_1, BC_5S_1 and BC_5S_2 offspring were artificially inoculated with WMV, CYSDV and *Px* races 1, 2 and 5 (isolates SF30, P-15.0 and C8, respectively). For the *Px* phenotyping assays, the maintenance of the different isolates and the inoculations were performed as described in (López-Martín *et al.*, 2022). BC_3S_1 and BC_5S_1 populations were inoculated with the physiological races 1, 2 and 5 during April-May. Whereas, due to availability of inoculum source and considering the response to the three races in TGR-1551 derived plant materials is homogeneous, BC_4S_1 and BC_5S_2 offspring were only inoculate with the race 5 isolate, the first of them during March and the later in October. In all cases, twelve days after inoculation, the infection was scored using a scale from 0 to 3, according to the level of sporulation of the fungus (López-Martín *et al.*, 2022).

CYSDV inoculations were carried out using *B. tabaci*, following the clip-cage method (Célix *et al*., 1996; Pérez-de-Castro *et al*., 2020). Virus symptoms were assessed once a week over a one-month period. The evaluation was based on the percentage of leaves showing typical CYSDV symptoms of infection. Additionally, virus titer was estimated by qRT-PCR (**Supplementary Table 2**).

The isolate WMV-Vera (MH469650) was used to mechanically inoculate the different families as well as TGR-1551 and BO at the one-to-two true leaf stage. WMV was mechanically inoculated as described by Pérez-de-Castro *et al*. (2019). Symptoms were evaluated at 15- and 30-days post-inoculation (dpi) with a visual scale from 0 (no symptoms) to 4 (severe mosaic and leaf distortion). Plants in classes 0 and 1 were considered as resistant. WMV infection was assessed by tissue printing at 15 and 30 dpi. Plant petioles of the younger leaves were cut and cross sections were bottled onto positively charged nylon membranes (Hybond-N, Amersham) that were further fixed by UV radiation (700 × 100 mJ/cm2) and hybridized with a digoxigenin-labeled RNA probe. This probe was constructed as described by Sáez *et al*., (2021), using the CP gene of WMV as insert. Prehybridization, hybridization and washing steps were performed as previously described by Sáez *et al*. (2021). Finally, CSPD (Roche Diagnostics) was used as

reagent for the chemiluminescent detection and films were exposed to the membrane for one hour.

4.4. BC_5S_2 field trial

In 2022, 3 selected BC_5S_2 families, TGR-1551, BO, F_1(TGRxBO) and `Carmen´ plants were grown in a field located in Museros (39°34'38.7"N 0°21'49.5"W, Valencia, Comunidad Valenciana). A randomized complete block experimental design with two blocks per accession, including 7 and 8 plants per block for the BC_5S_2 families and 3 plants per block for the rest of genotypes was used. Spacing between plants was 0.5m. All the ripe fruits were weighted to calculate the yield per plant. Five fruits per block were selected to be characterized and to study their acids and sugars profiles.

The presence of powdery mildew and viral symptoms was visually evaluated. Moreover, WMV and CYSDV titer were evaluated by qRT-PCR. Additionally, the presence of Moroccan watermelon mosaic *virus* (MWMV) and cucurbits aphids borne yellows virus (CABYV) was analyzed by PCR (**Supplementary Table 2**)

4.4.1. Melon fruits characterization

The collected fruits from the different offsprings were morphologically characterized. Five fruits per family and block (BC_4S_1, BC_5, BC_5S_1, BC_5S_2, F_{1PS}, BC_{1PS}, BC_{2PS}) and from their parental lines and controls (BO, PS, TGR-1551 and 'Carmen') were harvested at their commercial maturity state. The following traits were measured for each fruit: fruit weight (FW in kilograms, with digital scale), fruit length and diameter (FL, FD in cm, with graduated ruler), fruit shape index (FS, as the ratio of fruit length to fruit diameter), cavity diameter (CD, in cm, with a graduated ruler) cavity width (CW, in cm, as the ratio of the width of the seminal cavity to the fruit diameter), inner and external rind thickness (IRth, ERth in mm, with electronic digital caliper, I.C.T, S.L., La Rioja, España), rind and flesh firmness (RF, FF, measured as kg/cm^2 with a penetrometer, FT 327, with a plunger diameter of 8 mm, Alfonsine, Italy), flesh and rind color measured with a CR-400 colorimeter, Konica Minolta, Inc., Tokyo, Japan (coordinates Hunter Lab. L, a* and b*) (FCHl, FCa, FCb, RCHl, RCa, RCb) and soluble solids concentration (SSC) (measured as °Brix from drops of juice with a hand-held `Pocket´ refractometer (PAL-α), Atago CO., LTD, Tokyo, Japan). Flesh and rind firmness, color and total soluble solids were measured at two points in the equatorial region of the mesocarp.

4.4.2. Metabolomic analysis

At least 5 fruits from each studied family and their corresponding recurrent parental lines (BO and PS) were chosen at random to analyze their sugars and acids compound profiles. For this purpose, a 5 cm cross-section of the fruit´s equatorial area was cut, then the rind and the seeds were removed and the edible flesh was homogenized (Silent Crusher M; Heidolph, Schwabach, Germany) before it was frozen at -80°C until later analysis. Aliquots were further used to measure sugars (glucose, fructose and sucrose) and organic acids (citric, malic and glutamic acids) by capillary electrophoresis.

Sugar and acid analyses followed the methodology described by Martí *et al.* (2019) using an Agilent 7100 system (Agilent Technologies, Waldbronn, Germany). Samples were thawed in the dark, centrifuged at 510 g for 5 minutes, and the upper phase was diluted 1:20 with deionized water. The solution was then filtered through 0.22 µm membranes (Costar® Spin-X®, Corning, Amsterdam, The Netherlands). Fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) with dimensions of 50 µm internal diameter, 363 µm external diameter, 67 cm total length, and 60 cm effective length were employed for the separation process. Capillaries were conditioned before first use and flushed between samples and runs (Martí *et al*., 2019). Hydrodynamic sample injection, separation conditions, and absorbance measurements were performed according to Martí *et al*. (2019). Results were reported in g kg−1 of fresh weight, with sucrose equivalents (SE) calculated based on the sum of sugar contents weighed with their sweetening power (Koehler *et al.,* 1991)

4.5. Statistical analysis

The data obtained from the melon fruit characterization and the metabolomic analysis was used separately to perform the principal components analysis (PCA), using the function `prcomp' included in the package `stats´ v.4.2.2. of R (R Core Team, 2022). ANOVA tests and least significant different (LSD) test (p-value <0.05) were performed with the software STATGRAPHICS v.18.

DECLARATIONS

Competing interests

The authors declare that they have no competing interests

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Authors´contributions

Conceptualization, M.L.G.G., A.P.C., M.B.P., J.C.C and M.L.M; methodology, M.L.G.G., A.P.C., M.L.M., R.M, M.G.C, M.V and E.M.M.P; software, M.L. and J.C.C; investigation, M.L.G.G., M.L.M., J.C.C and A.P.C.; writing—original draft preparation, A.P.C., J.C.C. and M.L.M; writing—review and editing, M.L.G.G., M.B.P., J.C.C., M.L.M. and A.P.C.; funding acquisition, M.L.G.G., A.P.C and M.B.P. All authors have read and agreed to the published version of the manuscript.

Supplementary materials

The supplementary material can be found at:
https://drive.google.com/drive/folders/1hQZXVI0M2xnQBpihhgwnbtaABLNQlUOE?usp=sharing

Supplementary Table 1. Markers sets Background1, Background2, WMV1, WMV2, CYSDV1 and CYSDV2 used to genotyoe the studied families. The SNPs of the Background1 set have been retrieves from Perpiña *et al*. (2016). The SNPs of WMV1 and WMV2 sets have been retrieved from Pérez-de-Castro *et al.* (2019). The SNPs of CYSDV1 and CYSDV2 have been retrieved from Pérez-de-Castro *et al.* (2020). The physical position of the SNPs in versions v.3.6.1 and v.4.0 of the melon genome (available at https://melonomics.net/) is indicated. The SNPs (in brackets) and flanking sequences are included.

Due to its extent, Supplementary Table 1 can be found at: https://drive.google.com/drive/folders/1hQZXVI0M2xnQBpihhgwnbtaABLNQlUOE?usp=sharing

Supplementary Table 2. Primer sequences of the molecular markers used to genotype the different families studied (HRM markers) and to detected different viruses (RT-qPCR and PCR markers). The references from which the primers were retrieved are indicated.

PCR type	Marker name	Forward primer sequence	Reverse primer sequence	Reference
HRM	*cysdv14*	GAACAATCGTAAGTCCACTAAATCC	CCTGCAAATGTTGATCACG	Pérez-de-Castro *et al.* (2020)
HRM	*cysdv17*	AAGTCGAATGTCCGATTCTTC	CTCAAAAGAAGGCCAACTCC	Pérez-de-Castro *et al.* (2020)
HRM	*cysdv63*	TAAGAGGCTAACAGGACTGG	CTGTAGCTTCAACATTGAGTG	Pérez-de-Castro *et al.* (2020)
HRM	*cysdv65*	GAGATCTCTAAACAAATCAGAGG	TCATCCCTTTAACCAGAGTG	Pérez-de-Castro *et al.* (2020)
HRM	*b5wmv11*	TCCTGAATGGCCAATTTTTG	CTTTACACAATCCCGAATTTTTC	
HRM	*b11wmv10*	TGACCTGTAGCCAACTCACG	TGCCTGCCTGGGATATAATG	
HRM	*b11wmv11*	GCAGATGGAGCTGCTAAACC	CACAGAGCCAACAACTTTTCC	
RT-qPCR	qPCR-CYSDV	TGATGACGGGAAGGTTAGAGT	CTTCGGATCGGGTTGGACA	
RT-qPCR	qPCR-WMV	TGTTGCTTCATGGAAGATTGGT	AAAATTGTGCCATCAGGTGCTA	Chapter 3
PCR	MWMV-CP	GATCTTGCCTAGAGTCAGAG	CACTTACGCATGCCCAGGAG	Chapter 1
PCR	CABYV2316/2730	CTCCTTCCGATATTGGCTCG	CTCCAGTCAAARCCRGAGCAGTC	Desbiez *et al*. (2020)

Supplementary Table 3. Fruit characterization of the melons produced by the three pre-breeding lines obtained in this work, the pre-breeding line `Carmen´, their parental lines `Bola de Oro´(BO) and TGR-1551 and their offspring (F_1(TGRxBO)). FW: fruit weight; FS: fruit shape index; CW: cavity width; IRth, ERth: inner and external rind thickness; FF: flesh firmness; FCHl, FCa, FCb, RCHl, RCa, RCb: flesh and rind color (coordinates Hunter Lab. L, a* and b*); SSC: soluble solids concentration

Character	$BC_5(159\text{-}1)S_2$	$BC_5(159\text{-}2)S_2$	$BC_5(159\text{-}3)S_2$	BO	Carmen	F_1(TGRxBO)	TGR-1551
FW (Kg)	1.55±0.05	1.93±0.13	1.98±0.13	1.52±0.07	0.90±0.01	2.27±0.12	0.84±0.03
FS	1.19±0.01	1.69±0.03	1.73±0.04	1.27±0.06	1.23±0.02	2.30±0.05	1.49±0.02
CW (cm)	6.69±0.53	6.55±0.21	6.52±0.197	6.34±0.24	6.09±0.15	5.43±0.20	5.26±0.23
FF (Kg/cm^2)	2.24±0.09	1.67±0.10	2.67±0.12	2.29±0.21	2.32±0.18	7.22±0.34	9.54±0.75
Irth (mm)	3.94±0.29	4.76±0.26	4.93±0.09	3.19±0.22	3.11±0.24	1.63±0.85	0.66±0.35
Erth (mm)	0.36±0.025	0.49±0.03	0.42±0.03	0.46±0.04	0.50±0.03	0.38±0.02	0.37±0.03
FCHl	66.66±1.22	64.56±1.23	65.02±2.36	65.25±1.40	68.04±1.28	74.18±1.61	75.24±1.57
Fca	-6.62±0.64	-7.99±0.61	-7.38±0.37	-6.68±0.40	-7.35±0.67	-2.59±0.39	-1.57±0.12
FCb	14.65±0.63	16.14±0.52	15.35±0.42	14.16±0.47	14.45±0.68	8.13±0.49	5.77±0.27
RCHl	72.70±0.82	74.54±0.62	71.84±0.91	74.95±1.11	73.61±0.78	72.42±0.48	70.12±0.45
Rca	-8.14±0.82	-11.47±0.64	-8.85±0.47	-9.46±0.49	-9.92±0.32	-2.63±0.73	1.25±0.36
RCb	43.11±0.65	39.11±0.73	40.85±0.59	42.32±0.64	42.01±0.69	45.13±0.23	45.30±0.30
pH	5.90±0.06	5.95±0.05	5.80±0.11	5.90±0.10	6.00±0.00	4.80±0.32	4.95±0.36
SSC (°Brix)	12.00±0.40	11.31±0.30	11.22±0.22	12.33±0.34	12.33±0.49	5.44±0.17	5.58±0.16

Supplementary Table 4. Accumulation of sugars and acids in fruits from the three pre-breeding lines obtained in this work, the pre-breeding line `Carmen´, their parental lines `Bola de Oro´(BO) and TGR-1551 and their offspring (F_1(TGRxBO)). Results are reported in g kg^{-1} of fresh weight. TS: total sugars; SE: sucrose equivalents; ratio Hs: ratio hexose-sucrose.

	$BC_5(159\text{-}1)S_2$	$BC_5(159\text{-}2)S_2$	$BC_5(159\text{-}3)S_2$	BO	Carmen	F_1(TGRxBO)	TGR-1551
TS	89.7±5.36	92.51±2.62	93.73±1.82	115.72±3.38	112.6±3.88	29.43±0.92	30.85±1.66
SE	92.8±5.45	98.63±2.76	100.81±2.07	122.26±4.27	118.47±4.01	31.07±1.45	33.82±2.37
ratio HS	0.68±0.08	0.95±0.1	1±0.07	0.63±0.05	0.71±0.07	5±0	5±0
Glucose	22.12±0.59	26.06±0.58	26.86±0.84	26.22±1.26	27.53±0.7	20.04±0.29	19.73±0.56
Fructose	12.14±0.69	17.68±0.92	19.28±1.05	18.3±1.8	17.86±1.21	9.39±0.79	11.11±1.16
Sucrose	55.44±5	48.77±3.28	47.58±2.09	71.2±2.13	67.2±4.4	0±0	0±0
Citric acid	4.82±0.16	4.08±0.14	4.16±0.12	4.39±0.19	5.3±0.24	3.7±0.12	3.23±0.11
Malic acid	0.03±0.01	0.01±0	0.03±0	0.03±0.01	0.1±0.04	0.41±0.03	0.53±0.04

Supplementary Table 5. Fruit characterization of the melons produced by the different offspring derived from PS obtained in this work, the parental lines `Bola de Oro´(BO), `Piel de Sapo´(PS) and TGR-1551 and their offspring (F_1(TGRxBO)). Fruits obtained during the same year are indicated: (A) 2020; (B) 2021 and (C) 2022. FW: fruit weight; FS: fruit shape index; CW: cavity width; IRth, ERth: inner and external rind thickness; FF: flesh firmness; FCHl, FCa, FCb, RCHl, RCa, RCb: flesh and rind color (coordinates Hunter Lab. L, a* and b*); SSC: soluble solids concentration

A

	F_1-159-1	**F_1-159-2**	**BO**	**PS**
Year	2020	2020	2020	2020
FW (Kg)	1.47±0.13	0.95±0.13	1.77±0.07	1.93±0.14
FS	1.81±0.06	2.17±0.21	1.34±0.03	1.91±0.08
CW (cm)	4.44±0.16	3.4±0.26	5.97±0.16	4.39±0.11
FF (Kg/cm2)	2.23±0.14	5.6±1.32	3.14±0.48	1.71±0.17
Irth (mm)	3.86±0.28	2.76±0.32	3.96±0.22	3.84±0.16
Erth (mm)	0.48±0.03	0.68±0.12	0.61±0.07	0.66±0.06
FCHl	57.19±1.71	55.95±1.6	53.36±2.01	59.38±1.71
Fca	-4.47±0.39	-4.35±0.71	-4.46±0.41	-4.04±0.33
FCb	13.33±0.43	13.55±0.79	12.68±0.55	13.54±0.58
RCHl	43.77±1.29	45.25±2.15	67.89±0.86	34.38±1.04
Fca	-1.7±0.34	-2.24±0.54	7.01±0.96	-3.5±0.44
FCb	23.32±0.9	23.91±0.73	39.93±0.67	16.05±0.74
pH	5.91±0.06	6±0	6±0	6±0
SSC (ºBrix)	10.23±0.28	10.97±1.39	11.22±0.48	10.57±0.44

Supplementary Table 5. Continuation

B

	BC_1-159-1	BC_1-159-2	BO	PS	TGR-1551	F_1(TGRxBO)
Year	2021	2021	2021	2021	2021	2021
FW (Kg)	2.89±0.11	2.62±0.15	1.96±0.16	2.38±0.15	1.33±0.08	4.57±0.73
FS	1.79±0.07	1.88±0.04	1.32±0.01	1.63±0.02	1.54±0.06	2.1±0.09
CW (cm)	5.55±0.28	5.16±0.3	5.88±0.16	4.18±0.17	5.82±0.29	5.9±0.83
FF (Kg/cm2)	2.94±0.17	2.96±0.18	2.46±0.07	2.83±0.18	7.7±0.27	5.62±0.28
Irth (mm)	3.78±0.32	3.89±0.32	3.56±0.55	4.14±0.48	2.58±0.86	5.42±0.79
Erth (mm)	0.48±0.04	0.44±0.05	0.36±0.03	0.56±0.05	0.46±0.04	0.59±0.07
FCHl	56.18±0.76	56.75±1.77	52.33±1.12	59.34±1.44	73.16±1.56	72.83±1.94
Fca	-2.01±0.18	-3±0.36	-4.12±0.36	-2.2±0.1	-1.37±0.15	-1.21±0.14
FCb	10.23±0.36	10.62±0.6	12.71±0.67	10.09±0.2	8.22±0.37	8.93±0.32
RCHl	35.39±0.94	38.64±1.62	67.93±0.78	33.67±1.05	64.28±0.78	61.59±1.84
Fca	-1.95±0.22	-2.67±0.57	7.41±0.95	-2.3±0.54	17.51±1.4	12.49±1.53
FCb	16.49±0.68	19.87±1.07	39.51±0.45	16.57±0.78	38.63±0.54	35.67±1.38
pH	5.69±0.13	5.92±0.14	5.85±0.1	5.62±0.18	4±0	4.78±0.28
SSC (ºBrix)	12.54±0.37	12.4±0.51	10.88±0.38	12.45±0.44	5.65±0.19	5.92±0.3

Supplementary Table 5. Continuation

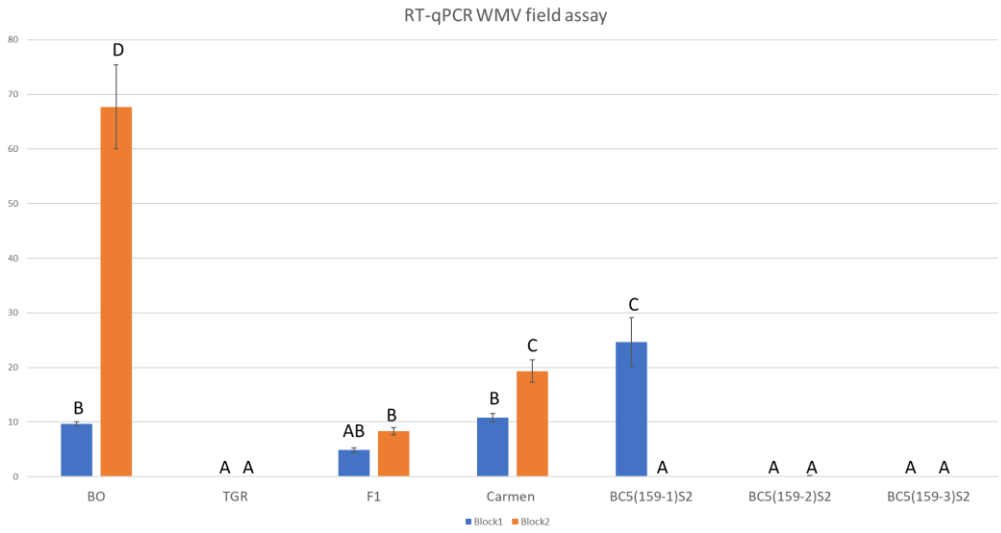
C

	BC_2-159-1-6	BC_2-159-1-4	BC_2-159-2-43	BO	PS
Year	2022	2022	2022	2022	2022
FW (Kg)	1.89±0.16	2.29±0.18	2.45±0.14	2.03±0.07	1.7±0.16
FS	1.68±0.05	1.83±0.04	1.84±0.05	1.34±0.02	1.78±0.07
CW (cm)	4.61±0.16	4.3±0.12	4.44±0.15	5.42±0.27	4.76±0.13
FF (Kg/cm2)	2.75±0.14	2.38±0.08	2.7±0.09	2.05±0.14	2.66±0.02
Irth (mm)	4.07±0.16	3.62±0.39	4.22±0.23	3.61±0.16	2.53±0.13
Erth (mm)	0.81±0.09	0.66±0.05	0.69±0.03	0.57±0.04	0.52±0.05
FCHl	57.31±2.27	59.16±2.05	57.16±1.17	53.51±2.11	70.82±2.5
Fca	-3.84±0.25	-5.01±0.41	-4.07±0.17	-5.2±0.37	-5.36±0.33
FCb	11.04±0.38	12.35±0.48	11.49±0.32	11.73±0.55	13.33±0.5
RCHl	51.6±2.98	48.81±1.72	42.04±1.25	72±0.57	40.48±1.77
Fca	-8.88±0.49	-8.58±0.37	-8.08±0.41	-3.2±0.75	-8.62±0.38
FCb	29.88±2.11	27.67±1.3	23.39±1.06	45.3±0.4	21.47±0.96
pH	6±0	6±0	6±0	6±0	6±0
SSC (ºBrix)	13.34±0.24	12.54±0.33	13.3±0.42	11.73±0.44	9.74±0.81

Supplementary Table 6. Accumulation of sugars and acids in fruits from the different offsprings obtained in this work, their parental lines `Bola de Oro´(BO) and `Piel de Sapo´(PS). Results are reported in g kg^{-1} of fresh weight. TS: total sugars; SE: sucrose equivalents; ratio Hs: ratio hexose-sucrose.

	Year	SE	TS	ratio HS	Glucose	Sucrose	Fructose	Citric acid	Malic acid	Glutamic acid
F_1-159-1	2020	81.88±3.03	75.75±2.84	0.57±0.04	13.62±0.61	48.88±2.99	13.25±0.56	3.66±0.23	0.37±0.11	0.07±0.02
BC_1-159-1	2021	96.83±2.75	100.02±2.91	0.33±0.01	14.31±0.62	73.05±2.05	9.47±0.51	3.95±0.11	0.07±0.02	0.03±0.01
BC_2-159-1-6	2022	126.15±4.23	124.19±4.17	0.34±0.03	20.74±1.24	93.4±3.71	10.05±0.84	1.99±0.07	1.18±0.11	0±0
BC_2-159-1-4	2022	120.13±8.54	117.64±8.53	0.52±0.1	24.94±1.7	80.41±10.41	12.29±1.56	2.08±0.13	0.76±0.16	0.03±0.02
F_1-159-2	2020	75.94±9.65	69.77±10.18	0.67±0.16	11.41±0.78	45.84±11.12	12.52±1.45	2.55±0.15	0.73±0.37	0.01±0.01
BC_1-159-2	2021	93.41±3.39	96.61±3.4	0.38±0.03	15.47±0.66	68.04±3.66	9.9±0.76	3.34±0.23	0.46±0.24	0.05±0.01
BC_2-159-2-43	2022	124.3±5.11	122.95±4.72	0.36±0.03	22.58±1.63	90.46±3.32	9.91±1.26	1.76±0.27	0.96±0.29	0±0
BO	2020	98.75±3.56	89.38±3.51	0.67±0.04	16.68±0.4	53.92±3.45	18.78±0.46	4.6±0.35	0.03±0.01	0±0
BO	2021	79.39±2	83.98±2.07	0.51±0.01	15.16±0.48	52.55±1.46	11.69±0.33	3.91±0.2	0.03±0	0.02±0.01
BO	2022	105.95±4.5	106.93±4.66	0.4±0.02	23.07±0.78	76.97±4.17	6.9±0.38	3.49±0.09	0.14±0.09	0±0
PS	2022	92.15±5.49	88.66±5.53	1.14±0.16	30.39±0.96	42.66±5.15	15.61±0.77	3.12±0.13	0.04±0.02	0±0
PS	2020	80.41±2.16	71.19±2.55	1.18±0.33	16.78±1.18	35.8±4.27	18.61±1.33	2.92±0.33	0.3±0.08	0.11±0.02
PS	2021	88.03±2.84	90.34±2.95	0.31±0.01	12.83±0.48	67.45±2.55	7.74±0.32	2.42±0.31	0.84±0.14	0.06±0.02

Supplementary Figure 1. Mean of watermelon mosaic virus titters measured by RT-qPCR. Different letters indicate significative differences (LSD test, p-value <0.05). Results obtained for plants of the same family/accession in different experimental blocks are indicated.



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General discussion

General discussion

Viral and fungal diseases are the main biotic stresses affecting cucurbit crops. Some of these diseases can cause yield reductions of up to 80%. Furthermore, this situation is exacerbated by international trade and increasing temperatures due to climate change, which favor the emergence of these pathogens in previously unaffected areas. In this context, conducting regular surveys in the main cucurbit-producing areas is crucial to comprehend the epidemiological status, monitor the evolution of prevalent viruses and fungi in different regions, and to identify emerging pathogens. Such studies promote the adaptation of breeding programs to meet the demands of farmers in the different production areas.

Regarding viral infections, these kinds of studies have been periodically carried out in other countries such as France and the United States. However, when this doctoral thesis started, the most recent epidemiological data collected in Spain dated back to the 2003-2006 campaigns and was restricted to Comunidad Valenciana and Murcia (Juarez *et al*., 2013; Kassem *et al.*, 2007). Therefore, a primary objective was set to provide an update on the incidence and genetic variability of viruses affecting the major cucurbit crops in the Spanish Mediterranean region. In parallel with the development of this doctoral thesis, several articles have been published addressing the same topic in Spain. However, these studies did not include Andalucía or Valencia and only examined the incidence of aphid-transmitted viruses (De Moya-Ruiz *et al*., 2021; Rabadán *et al*., 2021; Rabadán *et al*., 2023). Consequently, the information gathered in those works, along with the results obtained in this doctoral thesis, can be considered complementary, providing a comprehensive overview of the epidemiological situation in the main Spanish production areas.

In the studies conducted by Juarez *et al*. (2013) in Comunidad Valenciana during 2005-2006, it was observed that the virus with the highest incidence was CABYV, followed by WMV and PRSV, while ZYMV, MNSV, CMV, CVYV, and CYSDV had a lower incidence. Similarly, during 2003-2004 in Murcia (Kassem *et al*., 2007), CABYV was also the predominant virus, but there was also a high incidence of CYSDV and BPYV, while WMV, MNSV, CMV, ZYMV, CVYV showed infection rates similar to those observed in Valencia, and PRSV had a residual importance. Since these trials were conducted, several viruses have been reported for the first time in Spain. Among them, MWMV, ToLCNDV, CCYV, and a variant of CGMMV known as CGMMV-ES stand out (Crespo *et al.*, 2017; Juárez *et al*., 2014; Maachi *et al.*, 2022; Miras *et al.*, 2019)

The results obtained in this doctoral thesis showed slight changes in the epidemiological situation since the early 2000s. WMV and CABYV remain the predominant viruses in the studied areas, while the incidence of CMV has increased significantly. On the other hand, ZYMV and CYSDV, which previously showed a high infection rate in crops, were not detected in our trials. Additionally, CCYV and CGMMV, two of the emerging viruses in this territory, were neither detected, while the incidence of MWMV and ToLCNDV under conventional cultivation conditions was residual. However, different results were observed in a melon field cultivated under organic farming conditions, where the incidence of ToLCNDV rose dramatically. Furthermore, a high percentage of mixed infections was found in all surveyed fields. These results are consistent with observations from other surveys conducted during the same years in Spain and France, which showed the prevalence of aphid-transmitted viruses, specially WMV and CABYV (De Moya-Ruiz *et al*., 2021; Desbiez *et al*., 2020; Rabadán *et al*., 2021, 2023).

Regarding the genetic variability of the viral isolates found in different surveyed fields, the isolates of MWMV, ToLCNDV, and CABYV were grouped within their corresponding European cluster, which was consistent with previous studies (De Moya-Ruiz *et al*., 2021; Juárez *et al*., 2019; Rabadán *et al*., 2021). However, greater variability was observed for the CMV and WMV isolates analyzed. Phylogenetic analysis of the CMV isolates revealed that 19 out of the studied samples belonged to the IA-IA-IA group. To our knowledge, the last CMV phylogenetic analysis conducted in Spain was carried out during early 2000s (Sacristán *et al.*, 2004), showing the prevalence of IA-IA-IA isolates. On the other hand, the remaining 4 samples were classified as reassortant isolates IB-IB-IA. This type of isolates had been previously described in 1995 in eastern Spain but were not detected in subsequent studies (Bonnet *et al.*, 2005; Fraile *et al*., 1997; Sacristán *et al*., 2004). Moreover, nor have they been found in recent surveys conducted in other European countries (Desbiez *et al*., 2020).

With respect to the WMV samples, the sequencing of both NIb-CP and P3-CI regions unveiled a substantial molecular variability among the WMV isolates, establishing 7 novel profiles that had not been previously documented (Desbiez *et al*., 2020). The complete genome sequencing of each identified profile revealed a frequent occurrence of recombination among the isolates. This high frequency of recombination breakpoints had previously been observed when the “emerging” isolates were described for the first time (Desbiez & Lecoq, 2008) and also in recent studies (Desbiez *et al*., 2020). Furthermore, although nonsynonymous mutations

were not favored, several novel mutations were identified in Spanish isolates, particularly within the coding sequence of the P1 protein.

These findings demonstrate that despite the limited spread of newly emerging viruses in the Spanish Mediterranean basin, virus populations have the capacity to rapidly evolve through mutations and recombination/reassortment events. These evolutionary mechanisms, coupled with long-distance introductions via plant material exchanges and the influence of climate change, have the potential to trigger significant virus epidemics. Further studies should be conducted to determinate if the newly found isolates become prevalent or if they are able to overcome the previously described genetic resistances as it has previously happened with other viral isolates (Desbiez,*et al.,* 2021; Giner *et al*., 2017).

Concerning powdery mildew, to our knowledge, the last systematic monitoring assays conducted in Spain were carried out in the provinces of Málaga and Almería (Andalucía) during 1996-1999 growing seasons. In those assays four races (1, 2, 4 and 5) were detected (Del Pino *et al.*, 2002). Later, in 2008, race 3.5 was detected for the first time in Spain, concretely in Málaga (Torés *et al.,* 2009). One of the objectives of the research project in which this doctoral thesis has been carried out was to update the epidemiological situation of powdery mildew infections in Comunidad Valenciana, Región de Murcia and Andalucía. In this context, leaves infected with powdery mildew were collected from the same fields that were monitored for viral infections. Those samples were sent for the fungal classification to Dr. María Luisa Gómez-Guillamón (IHSM la Mayora, CSIC-UMA). The studies conducted by Dr. Gómez-Guillamón in these samples and other collected in different producing areas showed that races 2, 3.5 and 5 were present affecting melon in all the monitored areas. Moreover, a new race which is able to infect the genotypes resistant to race 3.5 was also observed in Cartagena (Región de Murcia). This race, usually referred to as 3.5+, had previously been observed by some breeding companies (personal communication). These results are consistent with those observed in other countries, such as Czech Republic, USA, South Korea….(Hong *et* al., 2018; Lebeda & Sedláková, 2006; McCreight & Coffey, 2011), where new and more virulent powdery mildew races have recently appeared.

In this sense, the current scenario for both fungal and viral infections in Spain and worldwide is quite similar. There is a dynamic state in which new and more virulent isolates and races continue to appear. This seriously threatens the sustainability of cucurbits cultivation.

Moreover, the genetic uniformity of the commercial varieties used in intensive horticulture promotes the spread of both viral and fungal pathogens. The implementation of efficient integrated control measures and improved crop management can lead to a reduction in infection incidence but it is not completely effective. Thus, the development of commercial varieties resistant to these pathogens is essential. There is a great genetic and phenotypic variability within the *C. melo* species. Indeed, various accessions, primarily belonging to the agrestis, acidulus, and conomon groups, represent invaluable material as sources of resistance alleles against viruses and fungi. However, comprehensive studies on the genetic and molecular bases regulating such resistances are necessary to harness them for the development of new resistant varieties. In this context, the main objective of this doctoral thesis was to develop melon resistant varieties to fungal and viral diseases.

The use of multi-resistant accessions allows for simplification of breeding programs, as a single donor parent can be used to introgress multiple sources of resistance into a single genetic background. In this regard, the African accession TGR-1551 (acidulus group) has been described as resistant to WMV, CYSDV, CABYV, MWMV, and races 1, 2, and 5 of powdery mildew. Moreover, it also carries the *Vat* gen and it is tolerant to *B. tabaci*. The genetic control of resistances derived from TGR-1551 against WMV, CYSDV, and powdery mildew had previously been described thanks to the development of F_2 and RILs populations derived from an initial cross between TGR-1551 and the susceptible cultivar BO (Díaz-Pendón *et al*., 2005; Lecoq & Desbiez, 2012; López-Sesé & Gómez-Guillamón, 2000; Palomares-Ríus *et al*., 2011, 2016; Yuste-Lisbona *et al*., 2010, 2011a, 2011b). However, these studies were mostly conducted using genetic maps based on AFLPs or SSRs markers, which had limited coverage. Consequently, the proposed candidate regions were too broad. Nevertheless, with the decreasing costs of sequencing technologies, it was possible to genotype by sequencing the RILs population. With that information, the COMAV´s cucurbits breeding group, in collaboration with Dr. Gómez-Guillamón, has recently been able to fine map the WMV resistance derived from TGR-1551 (Pérez-de-Castro *et al.*, 2019). This allowed the identification of SNP markers tightly linked to the resistance QTL that could be used in breeding programs. Two of the objectives of this doctoral thesis were to carry out similar studies to narrow down the candidate regions for the resistance to CYSDV and powdery mildew.

Even though CYSDV has not been detected in the surveys conducted in Spain, it is currently causing severe crop losses in the United States, Greece or Cyprus (Orfanidou *et al*., 2019; Tamang *et al.*, 2021), and it has recently been detected in

India or Saudi Arabia for the first time (Al-Saleh *et al*., 2015; Krishnan *et al.*, 2022). TGR-1551 is resistant to CYSDV and also tolerant to its vector, *B. tabaci*. The resistance mechanism against the virus in TGR-1551 has been demonstrated to involve restricting virus movement in the vascular system and/or preventing high levels of virus accumulation (Marco *et al.*, 2003). Initial segregation analyses in the progeny derived from crossing TGR-1551 with the susceptible Spanish cultivar 'Piel de Sapo' suggested a monogenic dominant control of the resistance (López-Sesé & Gómez-Guillamón, 2000). However, other studies proposed a codominant or more complex nature of the resistance (Park *et al.*, 2007).

In order to map the regions involved in the resistance to CYDSV derived from TGR-1551, the RIL population derived from the initial cross between TGR-1551 and BO was phenotyped for resistance to CYSDV in four independent trials. The phenotypic data from different environments exhibited a high correlation. Furthermore, a strong correlation was also observed between the viral load detected by RT-qPCR and symptom severity. The genotyping by sequencing of this population allowed for the identification of two QTLs on chromosome 5. The first QTL explained the variation in symptom severity, while the second QTL accounted for the variation in viral load as measured by RT-qPCR. Three of the RILs showing the highest resistance to CYSDV were selected to generate the corresponding BC_1S_1 generations. Evaluation of these families and their genotyping using 16 markers distributed on chromosome 5 (set CYSDV1) confirmed the results obtained with the RILs. Subsequently, a new set of 24 markers saturating the regions marked by the QTLs was designed (set CYSDV2), enabling further narrowing of the candidate intervals. Lastly, to complement the analysis of the RIL and BC_1S_1 populations and to assess the effect of introgressions on plants with BO genetic background, 200 plants from the BC_3 population derived from the cross TGR-1551 x BO were genotyped and phenotyped for resistance. Additionally, the BC_3S_1 offspring of 15 of these BC_3 plants were obtained. The analysis of these advanced populations confirmed the previously obtained results and further narrowed down the candidate interval to a 700 kb region.

Subsequent trials, that complement the results obtained in chapter 2, were conducted to determine the modifying effect of the minor QTL. These studies confirmed that this QTL alone is not capable of conferring resistance to CYSDV. However, in plants homozygous for the BO allele in the region of the major QTL and homozygous for the TGR-1551 allele in the minor QTL, a delay in symptom onset was observed, with slightly milder symptoms and a reduction in viral load. These

assays also confirmed that the major QTL is capable of conferring resistance to CYSDV by itself.

The accession PI 313970, that also belongs to the acidulus group but that was originated in India, has also been described as resistant to CYSDV. To study the genetic mechanisms underlaying the resistance derived from PI 313970, a $F_{2:3}$ population was phenotyped in field to natural infection (Tamang *et al.*, 2021). However, the presence of CCYV in the studied field made it impossible to determine which plants were susceptible or resistant, since PI 313970 is susceptible to CCYV and both viruses produce the same symptoms. Thereby, QTL mapping was assessed based on CYSDV viral titer. Two recessive QTLs were located on chromosomes 3 and 5, but the first of them was only observed in one of the field assays. Other resistance sources (PI 122847, PI 145594, PI 614185) also showed a recessive resistance control.

The resistance QTL located on chromosome 5 for PI 313970 co-localized with the minor QTL of TGR-1551 detected in this study between positions 20.124.095 – 24.125.662 (positions referred to as v.4.0 of the melon genome). This result supports the previously hypothesized notion that the resistances of TGR-1551 and PI 313970 are allelic and validates the findings obtained in this study. Further complementary studies should be conducted with controlled inoculations to determine the potential presence of another QTL in the PI 313970 accession that co-localizes with the major QTL of TGR-1551.

On the other hand, regarding the resistance to *Px* derived from TGR-1551, previous work had been done to map the regions involved in the resistance Initial studies conducted using an F_2 population genotyped with AFLPs, RAPDs, SSRs and CAPS markers indicated that the resistance to races 1, 2 and 5 was governed by a dominant-recessive epistasis (Yuste-Lisbona *et al*., 2010). Moreover, a QTL (*Pm-R5*), associated to the resistance to the three races had been mapped in chromosome 5. The presence of *Pm-R5* was further confirmed by analyzing the RIL population, which at this time was genotyped only with 260 SSR and EST-SSR markers. This assay also enabled the mapping of a QTL associated to the recessive gene on chromosome 12 (Beraldo-Hoischen *et al.*, 2012). However, when the molecular markers described as linked to the resistance genes were used in the breeding programs carried out by the research group, correlation between phenotype and genotype was not observed. This made us though that the previously defined region was not well defined. Hence, it was necessary to fine map the candidate regions. This has also been observed with other resistance sources, such as MR-1, EDISTO-47 or PMR5, where depending on the mapping population and the number

of markers used, the QTLs have been mapped in different positions (Natarajan *et al.*, 2016; Li *et al.*, 2017; Howlader *et al.*, 2020).

The availability of marker sets distributed throughout the melon genome, as well as specific marker sets for chromosome 5 previously used to map CYSDV resistance, facilitated the selection of BC_3 plants, whose progeny was used to narrow down the candidate intervals. Marker-assisted selection and analysis of advanced backcross generations allowed for the narrowing of the candidate region for resistance to races 1, 2, and 5 on chromosome 5 to 251 kb. The mapped region in this study did not co-localize with the previously proposed regions for TGR-1551. However, it did co-localize with the *qPx1-5* and *Pm-AN* QTLs linked to resistance against race 1 derived from MR-1 and Ano2, respectively. This region also co-localized with the major QTL previously associated to CYSDV resistance. The co-localization of CYSDV and powdery mildew resistances simplifies breeding programs, as it would only require selecting and introgressing a single region to confer resistance against both pathogens. This was observed when developing the pre-breeding line `Carmen´, where genetic selection for powdery mildew resistance also resulted in resistance to CYSDV (Palomares-Rius *et al*., 2018).

Most of the annotated genes within this region have resistance related functions. Among them, MELO3C004311 (TMV resistance protein N-like, a TIR-NBS-LRR gen), MELO3C004297 (Branched-chain-amino-acid aminotransferase-like protein), MELO3C002509 (Kinesin-like protein) and MELO3C002512 (exopolygalacturonase-like) are the principal candidate genes, since they have functions related to plant defense and cell-wall formation. Moreover, MELO3C004311 has also been proposed as the candidate gene, derived from MR-1, conferring resistance against *Px* race 1.

Regarding the recessive gene located on chromosome 12, the phenotyping and genotyping of the advanced backcrossed lines allowed to narrow the resistance interval to a 381 kb region (21.870.304 – 22.251.703). The narrowed region identified in this study does not overlap with the previously proposed resistance QTLs derived from TGR-1551 and falls outside the boundaries of candidate regions derived from other known resistance sources. Interestingly, the recessive gene found in TGR-1551 is distinct as, to our knowledge, no recessive genes conferring resistance to multiple Px races have been reported in melon before. This suggests that the resistance in TGR-1551 may be mediated by a gene different from those derived from other accessions. Most of the annotated genes have defense-related functions, which might indicate the presence of a resistance cluster.

Moreover, in this study, the phenotyping and genotyping analysis of the $BC_4(95)10S_1$ offspring revealed that the resistance conferred by the QTL on chromosome 5 decreased under low temperature conditions in heterozygous lines, while the resistance provided by the recessive QTL on chromosome 12 was not affected by temperature. These findings were in line with those reported by Beraldo-Hoischen *et al*. (2021), who observed that TGR-1551 and NIL21, which carries the *Pm-R* QTL derived from TGR-1551, were susceptible to powdery mildew when inoculated at low temperatures. In low temperature conditions, a delay in the resistance response was observed, and there was a decrease in the number of cells showing callose accumulation at the penetration site.

Further studies will be addressed to functional validate the candidate genes proposed for both CYSDV and powdery mildew resistances. However, the region of interest contains numerous genes with functions related to plant defense responses. To reduce the number of candidate genes, a strategy employed involves transcriptomic analysis. This approach aims to identify differentially expressed genes in TGR and BO in response to CYSDV or powdery mildew inoculations. Genes exhibiting differential behavior would be considered as primary candidates and subsequently validated using VIGS or CRISPR/Cas. The research group has previously employed this strategy. Transcriptomic analysis using RNA-seq was conducted after ToLCNDV inoculation of WM-7 and Piñonet (PS) accessions, which are resistant and susceptible to the virus, respectively (Sáez *et al*., 2022a). This analysis led to the identification of a primase gene responsible for resistance to this virus. Subsequently, the role of the primase in resistance against ToLCNDV and other geminiviruses was demonstrated through gene silencing and transient *Agrobacterium*-mediated expression (Sáez *et al*., 2022b; Siskos *et al*., 2023).

In the context of the research group's previous work, the candidate region responsible for WMV resistance was already fine-mapped (Pérez-de-Castro *et al.*, 2019). A major QTL associated to the recessive gene was mapped to a 140 kb region on chromosome 11 and an additional minor QTL with modifier effects was mapped on chromosome 5. Therefore, the next step in the study of WMV resistance, and one of the objectives of this doctoral thesis, was to investigate the transcriptomic changes induced by WMV in both TGR-1551 and BO.

In the TGR-1551 accession, the accumulation and spread of WMV were inhibited from early stages following inoculation, maintaining low levels throughout the plant life cycle. Conversely, in the susceptible cultivar BO, viral load at the onset of infection was low, but a significant increase in WMV accumulation occurred starting from 7 days post-inoculation, leading to the development of severe symptoms.

Similar findings have been reported in previous studies involving WMV (Gonzalez-Ibeas *et al.*, 2012)and various geminiviruses ; (Sáez *et al*., 2022). Consequently, we aimed to investigate the early defense response against the virus by comparing the transcriptomic changes in BO and the RIL-10-3, which was derived from the initial cross between TGR-1551 and BO and carried the resistance regions in chromosomes 11 and 5. The analysis revealed a substantial transcriptomic remodeling. A total of 616 genes and 7 gene clusters were found to be differentially expressed between the two accessions. This extensive alteration in gene expression had been previously observed in microarray experiments (Gonzalez-Ibeas *et al.*, 2012) and contrasts with the recessive nature of resistance. Among the overexpressed genes in TGR-1551, several were implicated in plant-pathogen interaction, hormone signaling transduction, MAPK pathways, and ubiquitination-mediated proteolysis. Activation of these defense systems, which are highly energy-demanding, may come at the expense of photosynthetic efficiency and basal metabolic pathways. Moreover, within the major QTL region on chromosome 11, three up-regulated DEGs were found. One gene encoded a basic 7S globulin-like protein (Bg7S), which, to our knowledge, had never been previously associated with virus resistance. Another gene coded for a dual specificity phosphatase 1, typically associated with higher resistance levels when repressed. The third gene encoded a mediator of RNA polymerase II transcription subunit 33A (MED33A), involved in plant defense and the regulation of the phenylpropanoid pathway, acting as a repressor. Since other mediator subunits (MED18, MED25) have been described as directly related to virus defense in *A. thaliana* (Hussein *et al.*, 2020) and the repression of the PPP can lead to broad-spectrum resistance (Desmedt *et al.*, 2021), we proposed MED33A as a candidate gene conferring resistance to WMV.

Sixteen DEGs were found within candidate region in chromosome 5, some of which had annotated functions previously reported as associated to resistance responses against pathogens. Among these genes there were ubiquitin family proteins, transmembrane proteins and importins, exoribonucleases, calreticulins, Trxs, calcium uptake proteins, GTP-binding proteins and serine-rich proteins. The significant transcriptomic remodeling observed in the minor QTL could be attributed to its positioning within a resistance cluster.

The results obtained in this study provide highly valuable information for understanding the processes involved in resistance against WMV in TGR-1551. Further VIGS and CRISPR/Cas assays will be carried out to functionally validate MED33A as the gene responsible of the resistance to WMV in TGR-1551.

Overall, the efforts made to fine map the QTLs implied in the resistance against CYSDV, WMV, and powdery mildew, as well as to understand the molecular mechanisms involved in those resistances, have yielded a set of valuable resources for breeding programs. TGR-1551 and BO accessions have been genotyped on multiple occasions using GBS and DArTseq, resulting in a large number of detected SNPs between them. These data, along with genotyping data from other wild species and landraces, have been used to create a panel of polymorphic markers distributed throughout the melon genome (Castro *et al.*, 2019; Esteras *et al*., 2013; López-Martín *et al.*, 2022; Perpiñá *et al.*, 2016). This set can be implemented on platforms such as Agena Bioscience iPLEX® Gold MassARRAY to facilitate genomic selection for the recovery of the recurrent parent's genetic background (Castro *et al.*, 2019; Perpiñá *et al.*, 2016;). Finally, the research derived from this doctoral thesis and previous works carried out by the Curucbit´s breeding group in collaboration with Dr. Gómez-Guillamón, has led to the development of HRM markers closely linked to the candidate regions for the different resistances. All the generated knowledge has been implemented in the breeding program to introgress the resistances to WMV, CYSDV and powdery mildew in the Yellow Canary `Bola de oro´ and the BGCM-126 `Piel de Sapo´ melon backgrounds. Both varieties belong to the ibericus group, which is widely consumed and produced in Spain and Portugal. This was the global objective of this doctoral thesis.

Five backcrosses followed by 2 self-pollination cycles were conducted to introgress the resistances to WMV, CYSDV and *Px* into the genetic background of BO. As a result, we obtained 3 pre-breeding lines named $BC_5(159\text{-}1)S_2$, $BC_5(159\text{-}2)S_2$ and $BC_5(159\text{-}3)S_2$. On average, these lines had recovered a 92.63% of BO genome. The pre-breeding lines were phenotyped for the resistance to the three pathogens in artificial inoculations, showing similar resistance levels to those observed for TGR-1551. Moreover, when they were tested in natural infection conditions were also found to be resistant to WMV; CYSDV and *Px* were not observed in the monitored field. As for the general appearance, all the pre-breeding lines had recovered the typical yellow rind color and the white-green flesh color. Attending internal and external fruit parameters, such as fruit shape, weight and color, $BC_5(159\text{-}1)S_2$ fruits were the more similar to BO, whereas $BC_5(159\text{-}2)S_2$ and $BC_5(159\text{-}3)S_2$ were bigger and heavier. In contrast, the previously developed pre-breeding line `Carmen´ produced smaller fruits (Palomares-Rius *et al*., 2018). However, when the acids and sugars contents were studied differences between the pre-breeding lines and BO were noticed. Main differences were due to a lower concentration of total sugars, sucrose and sucrose equivalents. The metabolic profile of the pre-breeding line

BC_5(159-1)S_2, which was more similar at the morphological level, differed when compared to BO, as showed lower glucose and fructose accumulations.

Regarding the PS breeding program, two BC_4(TGRxBO) plants, which carried the regions associated to resistance to WMV, CYDV and *Px*, were chosen as donor parents and the `Piel de Sapo´ melon type BGCM-126 was chosen as recurrent parent. To date, F_{1PS}, BC_{1PS} and BC_{2PS} fruits have been obtained and characterized. A huge advance in the breeding program has been observed through generations, reaching a phenotype highly similar to that of PS. Fruits heavier and more elongated than BO fruits, and also with a narrowed cavity, have been obtained over generations. Moreover, white flesh color and green rind color typical of PS fruits was already recovered in the F_1 and BC_1 offspring, respectively. Regarding the sugars profile, some BC_2 pre-breeding lines accumulated more sugars than the parental lines. This transgressive phenotype could be due to new allelic combinations between TGR-1551, BO and PS genetic backgrounds.

Eventhough a great recovery of the phenotypic characteristics of the parental lines has been obtained, further efforts should be made to get rid of undesirable TGR-1551 introgressions and also to narrow the introgressed resistance regions. Moreover, regarding the PS breeding program, it will be also necessary to select those plants that carry a higher percentage of PS genomic background in detriment of BO. In this sense, the research group has the necessary information to develop molecular markers for this purpose. Additionally, since aroma is an important quality parameter that greatly affects flavor, VOCs analysis should be also carried out. However, the pre-breeding lines obtained in this work constitute a valuable resource for breeders. These materials could be used as a tool for the introgression of WMV, CYSDV and powdery mildew resistances to landraces or elite melon breeding lines.

The research presented in this thesis represents a significant advance in the study of the key biotic factors affecting cucurbits cultivation in Spain, as well as in the progress of understanding the genomic regions and genes derived from the wild accession TGR-1551 involved in resistance against three important pathogens (WMV, CYSDV, and *Px*). In this regard, the evolution and cost reduction of sequencing technologies have been pivotal, allowing us to move from genetic maps with hundreds to thousands of markers, thus narrowing down the candidate resistance regions. This has also enabled thorough transcriptomic analyses to gain better insights into the response against the pathogens. While future functional validation experiments of candidate genes using VIGS or CRISPR/Cas will be necessary. The introgression programs of resistances into commercial genetic

backgrounds have validated the previously obtained results. Moreover, the generated pre-breeding lines can serve as a valuable resource for breeders, accelerating the breeding programs.

Conclusions

Conclusions

1. Conducting regular surveys in the main cucurbits producing areas is essential to comprehend the epidemiological status, monitor the evolution of prevalent viruses and to identify emerging pathogens. The surveys carried out during 2019 and 2020 showed that watermelon mosaic virus (WMV), cucurbits aphids borne yellow virus (CABYV), and cucumber mosaic virus (CMV) were the most important viruses affecting cucurbits in the Spanish Mediterranean basin. tomato leaf curl New Delhi virus (ToLCNDV) and Moroccan watermelon mosaic virus (MWMV) were also found in the monitored areas but at lower infection rates. These results were consistent with those obtained in other Spanish production areas. Breeding programs need to be adapted accordingly to meet the demands of farmers in different regions.

2. The sequencing of the detected isolates allowed the identification of new recombinant and reasortant isolates of WMV and CMV, respectively. Whereas low variability was found within CABYV, ToLCNDV and MWMV. Further studies will be addressed to determine if the newly detected isolates become prevalent in the different regions and if they are able to overcome the previously described resistances.

3. The African accession TGR-1551 (acidulus group) had previously been described as resistant to cucurbits yellowing stunting disorder virus (CYSDV), WMV and powdery mildew (*P. xanthii*) races 1, 2 and 5. Hence, it constitutes a valuable resource for breeding programs. However, it is essential to fine map the described resistances in order to be able to exploit this accession. The genotyping by sequencing and phenotyping for the resistance to CYSDV of a RILs population derived from the initial cross between TGR-1551 and the susceptible cultivar `Bola de Oro´ (BO), allowed the identification of two dominant QTLs associated to the resistance to CYSDV on chromosome 5. The major QTL was linked to the symptom's development. Whereas the minor QTL was linked to viral titer. Further studies carried out with advanced back-cross populations (BC_1S_1, BC_3 and BC_3S_1), derived from the same initial cross, confirmed the results previously obtained and allowed to narrow the candidate interval of the major QTL to a 700 kb region, where numerous resistance genes have been annotated.

4. The genotyping and phenotyping for the resistance to powdery mildew of advanced backcross populations derived from the initial cross between TGR-1551 and BO has allowed the fine mapping of the QTLs involved in the dominant-recessive epistasis governing this resistance. Thus, the recessive gene located on chromosome 12 has been narrowed to a 381 kb region; and the dominant gene, mapped on chromosome 5, was narrowed to a 251 kb region. Numerous resistance genes have been annotated in the narrowed intervals. Moreover, the resistance region on chromosome 5 co-localized with the major QTL linked to CYSDV resistance. The genes MELO3C004297 and MELO3C004311, located within the candidate interval on chromosome 5, have been proposed as candidate genes, since SNPs with a major modifying effect have been found in their sequences.

5. The transcriptomic analysis of BO and the RIL-10-3, derived from the initial cross between TGR-1551 and BO, allowed the characterization of the expression patterns involved in the resistance to WMV. It was noticed that a huge transcriptomic remodeling took place in the resistant line, which contrasts with the recessive nature of the resistance. The gen MELO3C021395, located on chromosome 11, which coded a mediator of RNA polymerase II transcription subunit 33A, has been proposed as the candidate gen conferring resistance to WMV.

6. The efforts made to fine map the QTLs implied in the resistances against CYSDV, WMV, and powdery mildew, as well as to understand the molecular mechanisms involved in those resistances, have yielded a set of valuable resources for breeding programs. The availability of both markers linked to the resistances, as well as sets of polymorphic markers distributed throughout the melon genome has facilitated the development of a breeding program to introgress these resistances into `Bola de Oro´ and `Piel de sapo´ commercial genetic backgrounds.

7. The BC_5S_2 pre-breeding lines obtained in the BO genetic background after the marker-assisted selection breeding program from the initial cross between TGR-1551 and BO are resistant to WMV, CYSDV and powdery mildew races 1, 2 and 5. They have also recovered the external and internal characteristics of BO, reaching good quality parameters. Moreover, a breeding program to introgress these resistances into a `Piel de Sapo´ genetic background has also been started. The obtained BC_{2PS} fruits have

recovered the external and internal characteristic of the recurrent parent and they even accumulate higher concentrations of sugars. The development of these pre-breeding lines demonstrates the usefulness of the markers linked to the resistance regions derived from TGR-1551 and constitute valuable resources for breeding purposes.

General references

General references

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