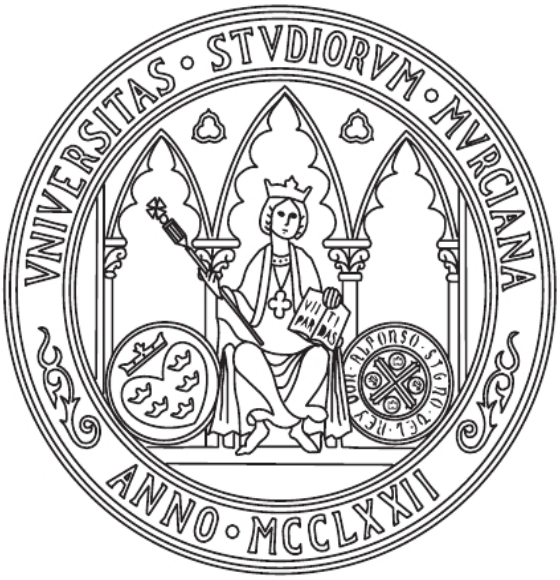

UNIVERSIDAD DE MURCIA

DEPARTAMENTO DE GENÉTICA Y MICROBIOLOGÍA

Muestreo y análisis del transcriptoma de melón (*Cucumis melo* L.)

D. Daniel González Ibeas

2012

UNIVERSIDAD DE MURCIA
Facultad de Biología
Departamento de Genética y Microbiología

CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS
Centro de Edafología y Biología Aplicada del Segura
Departamento de Biología del Estrés y Patología Vegetal

Muestreo y análisis del transcriptoma de melón (*Cucumis melo* L.)

Tesis Doctoral

Daniel González Ibeas
2012

UNIVERSIDAD DE
MURCIA

D. Francisco Murillo Araújo, Catedrático en la Universidad de Murcia, en el Departamento de Genética y Microbiología, INFORMA:

Que la Tesis Doctoral titulada "Muestreo y análisis del transcriptoma de melón (*Cucumis melo* L.)", ha sido realizada por D. Daniel González Ibeas, bajo la inmediata supervisión de Miguel Aranda Regules y Verónica Truniger Rietman, y que el Departamento ha dado su conformidad para que sea presentada ante la Comisión de Doctorado.

En Murcia, a 16 de Mayo de 2012

Fdo. Francisco Murillo Araújo

A QUIEN CONCIERNA

Miguel A. Aranda Regules, Profesor de Investigación del CEBAS-CSIC y Veronica Truniger Rietman, Investigadora Científica del CEBAS-CSIC, ambos directores de la tesis doctoral de Daniel González Ibeas,

INFORMAN:

Daniel González Ibeas ha trabajado en nuestro laboratorio durante los últimos siete años. Durante este periodo, ha desarrollado su labor de investigación en el marco de una serie de proyectos encaminados a desarrollar herramientas de genómica en melón para el estudio de caracteres de resistencia a patógenos. En una primera fase, generó un conjunto de genotecas de cDNA normalizadas a partir de varios tipos de tejidos de melón (infectados y sanos) y secuenció un conjunto muy amplio de ESTs de estas genotecas, creando así la primera colección importante de secuencias de melón (Gonzalez-Ibeas et al., 2007). A continuación, siguiendo un esquema muy parecido, generó otro conjunto de genotecas de pequeños RNAs de melón que secuenció y analizó, creando la primera lista comprensiva de pequeños RNAs de melón (Gonzalez-Ibeas et al., 2011). La colección de ESTs de melón mencionada se ha usado para generar un microarray específico para esta especie que el doctorando utilizó para analizar un carácter de resistencia a virus en melón (Gonzalez-Ibeas et al., 2012a). Para este último trabajo, debió realizar una puesta a punto metodológica que resultó en la generación de resultados que también se han publicado (Gonzalez-Ibeas et al., 2012b).

Así pues, el conjunto de las cuatro publicaciones arriba mencionadas forman un bloque de resultados homogéneo, y constituyen el cuerpo principal de la tesis doctoral de Daniel González Ibeas, titulada "Muestreo y análisis del transcriptoma de melón (*Cucumis melo*)". Por esta razón, consideramos oportuno presentar la memoria de la tesis como el compendio de las publicaciones mencionadas. Los artículos siguen obviamente una unidad temática, y abarcan muy sobradamente el trabajo necesario que justifica la formación de un doctorando.

Artículos:

DANIEL GONZALEZ-IBEAS, J. BLANCA, C. ROIG, M. GONZÁLEZ-TO, B. PICÓ, V. TRUNIGER, P. GÓMEZ, W. DELEU, A. CANO-DELGADO, P. ARÚS, F. NUEZ, J. GARCIA-MAS, P. PUIGDOMÈNECH, M. A. ARANDA (2007). MELOGEN: an EST database for melon functional genomics. **BMC Genomics,** 8, 306.

DANIEL GONZALEZ-IBEAS, JOSÉ BLANCA, LIVIA DONAIRE, MONTSERRAT SALADIÉ, ALBERT MASCARELL-CREUS, ANA CANO-DELGADO, JORDI GARCIA-MAS, CESAR LLAVE, MIGUEL A. ARANDA (2011). Analysis of the melon (Cucumis melo) small RNAome by high-throughput pyrosequencing. **BMC Genomics**, 12:393.

DANIEL GONZALEZ-IBEAS, JOAQUIN CAÑIZARES, MIGUEL A. ARANDA (2012a). Microarray analysis shows that recessive resistance to Watermelon mosaic virus in melon is

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associated with the induction of defense response genes. **Molecular Plant-Microbe Interactions**, 25:107-118.

DANIEL GONZALEZ-IBEAS, JOAQUIN CAÑIZARES, JOSÉ BLANCA, VERÓNICA TRUNIGER, MIGUEL A. ARANDA (2012b). A cost-effective double-stranded cDNA synthesis for plant microarrays. **Plant Molecular Biology Reporter**, DOI: 10.1007/s11105-012-0427-5.

Y para que así conste, firmamos el presente escrito en Murcia, a 7 de marzo de 2012.

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Vista la solicitud presentada el día 16 de abril de 2012, por D. DANIEL GONZÁLEZ IBEAS, con DNI número 52.686.283-E, sobre autorización para presentación de tesis doctoral como compendio de publicaciones con carácter previo a la tramitación de la misma en la Universidad de Murcia, le comunico que la Comisión de General de Doctorado, vistos:

- el informe previo del Departamento de Bioquímica y Biología Molecular A, responsable de la autorización de la tesis doctoral en fase de elaboración, de esta Universidad, y
- el visto bueno de la Comisión de Ramas de Conocimiento de Ciencias ,

resolvió, en su sesión de 15 de mayo de 2012, **ACCEDER** a lo solicitado por el interesado pudiendo, por lo tanto, presentar su tesis doctoral en la modalidad de compendio de publicaciones.

Lo que en cumplimiento del artículo 58 de la vigente Ley 30/1992, de Régimen Jurídico de las Administraciones Públicas y del Procedimiento Administrativo Común, de 26 de noviembre, se **notifica a** D. DANIEL GONZÁLEZ IBEAS, significándole que contra esta resolución, que pone fin a la vía administrativa, se podrá interponer potestativamente ante el mismo órgano que la ha dictado, recurso de reposición, en el plazo de un mes a contar desde el día siguiente a su notificación, de acuerdo con lo dispuesto en el art. 116 de la citada Ley.

Si no hiciera uso del recurso de reposición podrá interponer recurso contencioso-administrativo, en el plazo de dos meses desde la notificación de este acuerdo, en la forma establecida en la Ley 29/1998, de 13 de julio, reguladora de dicha Jurisdicción.

Murcia, 15 de mayo de 2012
Vicerrectora de Estudios y
Presidenta de la Comisión General de Doctorado

UNIVERSIDAD DE MURCIA Vicerrectorado de Estudios

Concepción Palacios Bernal

Agradecimientos

Agradezco la ayuda y apoyo de todas las personas que han contribuído a hacer posible la realización de esta Tesis. Gracias a César LLave y Livia Donaire por acogerme en su grupo. Gracias a todas las personas que formaron parte del Consorcio de Genómica Funcional Español, con especial mención a Jose Blanca y Joaquín Cañizares. Gracias a César Flores y María José López, de los servicios centrales de la Universidad de Murcia. Gracias a los componentes del Departamento de Genética de la Universidad de Murcia por permitirme recibir unos cursos de doctorado de excelencia, con especial mención a Francisco Murillo. Y gracias a todas las personas que han formado parte del grupo de Patología Vegetal del CEBAS, donde he realizado la Tesis, por su apoyo, en especial a mis directores, Miguel Aranda y Veronica Truniger.

Índice

Resumen general y presentación de las publicaciones

El melón (*Cucumis melo*) es uno de los frutos carnosos destinados a consumo en fresco más importantes del mundo, y su cultivo es importante en regiones templadas, tropicales y subtropicales del planeta. Su producción en el año 2004 excedió los 874 millones de toneladas métricas en todo el mundo, y los 25 millones en España, convirtiendo nuestro país en el quinto productor mundial y el primero en Europa (FAOSTAT). Las infecciones por virus son una de las principales causas de pérdidas de producción y de calidad de los frutos de melón; en particular, existen numerosos virus de RNA que afectan los cultivos de melón (Oerke y Dehne 2004; Woolhouse et al. 2005). La condición de los virus de parásitos intracelulares, debido a no disponer de una maquinaria metabólica propia, complica el diseño de productos antivirales aplicados directamente sobre la planta. Así pues, el cultivo de varidades de melón genéticamente resistentes a virus es una de las principales estrategias para previnir este tipo de infecciones (Kang et al. 2005; García-Arenal y McDonald 2003). La información genética de una especie vegetal proporciona un punto de partida para la generación de herramientas a usar en la mejora de dicha especie en varios aspectos, por ejemplo en la resistencia a virus y otros patógenos, o en la calidad de fruto. El análisis y muestreo del contenido total de transcritos celulares de RNA (transcriptoma) mediante las colecciones de secuencias que se expresan (de sus siglas en inglés *expressed sequence tags*, ESTs) ha sido ampliamente utilizado enpara dar un primer abordaje funcional al contenido genético de un organismo. Se han llevado a cabo varios trabajos de este tipo en especies de interés agronómico (Ouyang y Buell 2004; Newcomb et al. 2006; Silva et al. 2005; Forment et al. 2005), pero a pesar de la importancia del melón y de lo expuesto anteriormente, cuando el trabajo de esta Tesis comenzó, había depositadas en las bases de datos de ESTs un número muy bajo de secuencias. Se han cosntruído 8 genotecas normalizadas de DNA complementario (cDNA) a transcritos celulares poliadenilados a partir de varios tejidos de melón, incluyendo raíz, fruto, hoja y cotiledón, para su posterior secuenciación y generación de una base de datos con más de 30.000 ESTs, cuyos resultados sientan las bases del trabajo descrito en la Tesis (Gonzalez-Ibeas et al. 2007). A partir de la información generada se ha construído un chip o microarray de DNA con más de 17.000 secuencias consenso únicas (unigenes) que se expresan en melón (Mascarell-Creus et al. 2009). El microarray se ha usado para analizar la respuesta transcriptómica de plantas de melón infectadas con un virus de RNA de interés agronómico, el virus del mosaico de la sandía (*Watermelon mosaic virus*, WMV; género: *Potyvirus*; familia: *Potyviridae*), cuyos resultados se plasman en la Publicación I.

Además de los transcritos que codifican proteínas analizados en el microarray, la porción del transcriptoma compuesta por los RNAs no codificantes es de gran interés en la comunidad científica, en particular la que corresponde a pequeños RNAs, por su implicación en numerosos procesos celulares (Vaucheret 2006). En el caso del trabajo con virus de RNA de plantas, su estudio cobra especial interés porque pequeños RNAs derivados del genoma de virus se generan como parte de la respuesta defensiva de la planta mediada por silenciamiento génico (Llave 2010). La Publicación II describe la construcción de 10 genotecas de pequeños RNAs a partir de varios tejidos de melón, incluyendo fruto, ovario y cotiledones infectados con WMV y con el virus de las manchas necróticas del melón (*Melon necrotic spot virus*, MNSV; género: *Carmovirus*; familia: *Tombusviridae*). La aproximación seguida durante la construcción de las genotecas ha permitido muestrear tanto los pequeños RNAs endógenos de la planta, como los exógenos derivados a partir del genoma viral en las muestras infectadas.

Con las herramientas moleculares y bioinformátcas generadas durante el trabajo se ha llevado a cabo una puesta a punto metodológica en la generación de cDNA de doble acadena para hibridaciones ne microarrays (Publicación III). Se ha optado por presentar la Tesis en formato de compendio de publicaciones. El trabajo queda estructurado en torno a las tres publicaciones (I, II y III) que se enumeran a continuación, describiendo la aportación del doctorando en cada una de ellas.

Publicación I. Gonzalez-Ibeas, Daniel, Joaquin Cañizares, y Miguel Aranda. 2012. “Microarray analysis shows that recessive resistance to Watermelon mosaic virus in melon is associated with the induction of defense response genes.” *Molecular Plant-Microbe Interactions* 25 (1): 107-118. doi:10.1094 / MPMI -07-11-0193.

Aportación del doctorando: Cultivo de plantas de melón, inculación del virus en cotiledones, extracciones de RNA y procesado de las muestras. Análisis bioinformático de los resultados de microarray bajo tutela de Joaquín Cañizares (COMAV, Valencia). Diseño experimental y escritura del manuscrito bajo tutela de Miguel A. Aranda.

Publicación II. Gonzalez-Ibeas, Daniel, José Blanca, Livia Donaire, Montserrat Saladié, Albert Mascarell-Creus, Ana Cano-Delgado, Jordi Garcia-Mas, Cesar Llave, y Miguel Aranda. 2011.

"Analysis of the melon (Cucumis melo) small RNAome by high-throughput pyrosequencing." *BMC Genomics* 12: 393. doi:10.1186/1471-2164-12-393

Aportación del doctorando: Cultivo de las plantas de melón e inoculación del virus en las muestras infectadas con el virus del mosaico de la sandía. Elaboración de las 12 genotecas de pequeños RNAs bajo tutela de César LLave Correas (CIB, Madrid). Análisis bioinformático de los resultados de secuenciación bajo tutela de Jose Blanca Postigo (COMAV, Valencia). Diseño experimental y escritura del manuscrito bajo tutela de Miguel A. Aranda.

Publicación III. Daniel Gonzalez-Ibeas, Jose Blanca, Joaquin Cañizares, Veronica Truniger, y Miguel A. Aranda. 2012. "A cost-effective double-stranded cDNA synthesis for plant microarrays". *Plant Molecular Biology Reporter*, First on line. doi: 10.1007/s11105-012-0427-5

Aportación del doctorando: Cultivo de las plantas de melón y preparación de las muestras de RNA. Síntesis de cDNA de doble cadena por los dos procedimientos descritos en la publicación. Análisis bioinformático de los resultados de microarray bajo tutela de Joaquín Cañizares y Jose Blanca Postigo (COMAV, Valencia). Diseño experimental y escritura del mansucrito bajo tutela de los directores de Tesis.

El trabajo realizado durante la Tesis ha dado lugar también a otras publicaciones donde el doctorando figura como coautor en colaboraciones y que se detallan a continuación.

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Publicación I.

Título: Microarray analysis shows that recessive resistance to *Watermelon mosaic virus* in melon is associated with the induction of defense response genes

Referencia completa: Gonzalez-Ibeas, Daniel, Joaquin Cañizares, y Miguel Aranda. 2012. "Microarray analysis shows that recessive resistance to *Watermelon mosaic virus* in melon is associated with the induction of defense response genes." *Molecular Plant-Microbe Interactions* 25 (1): 107-118. doi:10.1094 / MPMI -07-11-0193.

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Aportación del doctorando: Cultivo de plantas de melón, inculación del virus en cotiledones, extracciones de RNA y procesado de las muestras. Análisis bioinformático de los resultados de microarray bajo tutela de Joaquín Cañizares (COMAV, Valencia). Diseño experimental y escritura del manuscrito bajo tutela de Miguel A. Aranda.

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Microarray Analysis Shows That Recessive Resistance to *Watermelon mosaic virus* in Melon Is Associated with the Induction of Defense Response Genes

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Resistance to *Watermelon mosaic virus* (WMV) in melon (*Cucumis melo* L.) accession TGR-1551 is characterized by a significant reduction in virus titer, and is inherited as a recessive, loss-of-susceptibility allele. We measured virus RNA accumulation in TGR-1551 plants and a susceptible control ('Tendral') by real-time quantitative polymerase chain reaction, and also profiled the expression of 17,443 unigenes represented on a melon microarray over a 15-day time course. The virus accumulated to higher levels in cotyledons of the resistant variety up to 9 days postinoculation (dpi) but, thereafter, levels increased in the susceptible variety while those in the resistant variety declined. Microarray experiments looking at the early response to infection (1 and 3 dpi), as well as responses after 7 and 15 dpi, revealed more profound transcriptomic changes in resistant plants than susceptible ones. The gene expression profiles revealed deep and extensive transcriptome remodeling in TGR-1551 plants, often involving genes with pathogen response functions. Overall, our data suggested that resistance to WMV in TGR-1551 melon plants is associated with a defense response, which contrasts with the recessive nature of the resistance trait.

Virus resistance in plants may involve the activation of a resistance response, the inhibition of virus functions, or the loss of virus susceptibility. Dominant resistance is normally associated with the activation of resistance responses (Marathe et al. 2004), although specific examples involve the inhibition of a viral function (Ishibashi et al. 2007). In contrast, recessive resistance is usually associated with the loss of susceptibility (Truniger and Aranda 2009). Several cultivar-specific recessive resistance genes have been cloned and characterized, showing that they encode eukaryotic translation initiation factors (eIF) of the 4E and 4G families (Robaglia and Caranta 2006; Truniger and Aranda 2009). Where resistance mechanisms have been dissected in detail, and it has been shown that recessive alleles are unable to provide a complementary function for the virus. For example, the eIF4E^{Leu228} protein encoded by the melon *nsv* resistance gene is unable to complement the cap-independent translational initiation of *Melon necrotic spot virus* RNAs (Nieto et al. 2006; Truniger et al. 2008). However, the characterization of artificially induced mutants has shown that not all loss-of-susceptibility alleles correspond to eIF4E or eIF4G genes and, therefore, that other recessive virus-resistance genes may exist (Truniger and Aranda 2009). The characterization of these resistance genes and their corresponding mechanisms may provide insight into undiscovered biological processes that confer virus resistance in plants. In the absence of a complete molecular characterization of such resistance genes, the comparative analysis of diverse physiological characteristics in virus-inoculated susceptible and resistant plants might shed light on the resistance mechanisms (Palukaitis and Carr 2008).

Viruses affect the physiology and metabolism of infected plants, and this can be monitored by looking for changes in host gene expression profiles that are either directly or indirectly influenced by viral infection (Aranda et al. 1996; Maule et al. 2002; Wang and Maule 1995). With the advent of high-throughput technologies such as expressed sequence tags (EST), microarrays, and next-generation sequencing, it has become feasible to provide functional data for many genes simultaneously and to study the remodeling of the plant transcriptome in response to virus infections (Whitham et al. 2006). Over the last 10 years, DNA microarrays have become a popular strategy for comparative high-throughput gene expression analysis. The results of more than 500,000 experiments have been deposited in the Gene Expression Omnibus database (GEO) (Barrett et al. 2011), and many tools are available for mining the data (Dudoit et al. 2003; Page and Coulibaly 2008).

Microarrays have been used to monitor changes in the transcriptome in response to virus infections in both compatible (Golem and Culver 2003; Senthil et al. 2005; Whitham et al. 2003) and incompatible (Marathe et al. 2004) interactions. This strategy has focused on commercially important crops such as grapevine (Espinoza et al. 2007), citrus fruit (Gandía et al. 2007), potato (Gammelgård 2007; Pompe-Novak et al. 2005), rice (Satoh et al. 2010), populus (Smith et al. 2004), maize (Shi et al. 2005), soybean (Babu et al. 2008), and tomato (Catoni et al. 2009). The relative scarcity of genomic data for melon has made such an approach more challenging in this species but the number of available melon EST has increased considerably in the last 4 years (Clepet et al. 2011; Gonzalez-Ibeas et al. 2007). In the case of the Melogen database, more than 33,000 EST have been sequenced to generate approximately 17,000 tentative consensus sequences (unigenes) (Gonzalez-Ibeas et al. 2007). A publicly available database containing all EST, contig images, and several tools for data analysis and mining has been created, and the unigene sequences have been used to

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*The *e*-Xtra logo stands for "electronic extra" and indicates that four supplementary figures and two supplementary tables are published online.

construct an oligo-based DNA microarray with a basic four-plex design and 75,000 probes. Each unigene is represented by four 60-mer probes, synthesized by photolithography, and designed according to quality rules based on uniqueness (non-redundancy), frequency in the transcriptome and melting temperature. This platform has been validated and used to analyze fruit quality traits, ovary development, and pathogen infections (Mascarell-Creus et al. 2009).

Here, we describe microarray experiments involving 17,443 unigenes represented on the melon microarray, which reveal extensive remodeling of the melon transcriptome in resistant and susceptible genotypes in response to infection with *Watermelon mosaic virus* (WMV) (genus *Potyvirus*, family *Potyviridae*). The resistant TGR-1551 accession is either asymptomatic or exhibits mild disease symptoms following mechanical inoculation with WMV, whereas susceptible cultivars show severe mosaic symptoms in systemically infected leaves. Infected TGR-1551 plants also have a substantially lower virus titer than susceptible controls (Diaz-Pendon et al. 2005). The inheritance of the resistance trait is thought to be controlled by a recessive allele, perhaps in combination with an epistatic interaction at a second locus (Diaz-Pendon et al. 2005). We describe the differentially expressed unigenes revealed by the microarray experiments and their relevance in terms of the observed physiological responses to infection, and discuss potential mechanisms controlling TRG-1551 resistance to WMV.

RESULTS

Accumulation of virus RNA in resistant and susceptible melon plants inoculated with WMV.

The progress of WMV infection in the resistant accession TGR-1551 and the susceptible 'Tendral' was determined by comparing the accumulation of WMV RNA by real-time quantitative polymerase chain reaction (qPCR) in inoculated cotyledons at different times after inoculation, and in systemically infected leaves. WMV accumulated to higher levels in TGR-1551 cotyledons up to 9 days postinoculation (dpi) but the situation had reversed by 15 dpi (Fig. 1A). In the systemically infected second true leaf, WMV accumulated to much higher levels in 'Tendral' than TGR-1551 by 15 dpi (Fig. 1A). No symptoms were evident in the cotyledons of either genotype or in systemically infected TGR-1551 leaves, whereas severe symptoms were observed in infected 'Tendral' leaves, including mosaics, vein banding, leaf bubbling or malformation, and growth stunting (Fig. 1B). These results confirmed previous observations (Diaz-Pendon et al. 2005) and showed that TGR-1551 resistance to WMV manifests as a significant virus titer reduction in systemically infected leaves accompanied by an almost complete absence of symptoms. For the transcriptomic analysis described below, we used samples from inoculated cotyledons at 1 and 3 dpi to monitor early changes in response to virus infection, and samples from inoculated cotyledons at 7 dpi because, at this time, there was a significant difference in virus accumulation between the genotypes, completing a temporal window with three time points. In addition, samples from systemically infected leaves at 15 dpi were also included in the analysis (Fig. 1A).

Construction of microarray expression data sets, sources of variability, and the amplitude of deregulation.

We used a previously validated melon microarray (Mascarell-Creus et al. 2009) to carry out 36 hybridizations on cotyledon samples (two genotypes × two treatments × three sampling times × three biological replicates) and 12 for leaf samples (two genotypes × two treatments × three biological replicates). Microarray hybridization images were reconstructed for visual inspection using raw expression data and we did not detect artifacts such as scratches, bubbles, or high local or overall background. Expression data were normalized as previously described (Mascarell-Creus et al. 2009).

In order to characterize the global biological variability among samples, normalized data were processed by principal component analysis (PCA). Cotyledon samples were primarily grouped by genotype (TGR-1551 versus 'Tendral') and then by days postinoculation. No obvious association was found among infected versus mock-inoculated samples, except perhaps for TGR-1551 cotyledons at 3 and 7 dpi (Fig. 2A). Therefore, transcriptomic alterations over time or genotype appeared to be more important than alterations associated with viral infection in inoculated cotyledons. Intriguingly, more dispersion was observed in the TGR-1551 samples, particularly the mock inoculations. Indeed, three of them were considered outliers and, therefore, were excluded from further analysis. To use the same criterion for both genotypes, three mock-inoculated cotyledon samples from each of the three time points were also excluded from the analysis, reducing the group of samples used for further analysis to three biological replicates for infected samples and two biological replicates for mock-inoculated cotyledons. Infected versus mock-inoculated leaves in each principal group showed limited differentiation in 'Tendral' samples but extensive differentiation in TGR-1551 samples, suggesting a significant transcriptomic impact in leaves of the resistant genotype where a clear differentiation among infected versus mock-inoculated samples was observed (Fig. 2B). Higher variability was found among the 'Tendral' biological replicates. To confirm these PCA results, a hierarchical clustering analysis was performed on the samples using the expression data after normalization (Fig. 2C and D). In cotyledon samples, principal clusters were identified first by genotype. In agreement with the PCA results, 'Tendral' samples grouped first by time instead of the presence or absence of infection, confirming that transcriptomic variation over time was more important than the response to infection in this genotype. In contrast, TGR-1551 cotyledon samples from 3 and 7 dpi grouped depending on whether or not they were infected (Fig. 2C). In conclusion, transcriptomic remodeling due to WMV infection appears to have more profound effects in TGR-1551 than in 'Tendral' cotyledons, a phenomenon much more pronounced in the leaf samples where small transcriptomic changes and replicate dispersion in 'Tendral' led to mixed clustering of mock-inoculated and infected samples (Fig. 2D).

Genes differentially expressed in inoculated cotyledons.

Differentially expressed unigenes in cotyledons were identified using microarray significant profiles (MaSigPro) (Conesa et al. 2006). The MaSigPro program is based on regression model approaches and is a good alternative to classical methods based on *t* tests, such as significance analysis of microarrays (SAM) (Tusher et al. 2001) or analysis of variance-based methods (Park et al. 2003), for the analysis of time course microarray series. This is because regression-based methods are considered more effective for capturing the dynamic nature of time course data (Conesa et al. 2006). Using time as a continuous variable and a false discovery rate (FDR) of 0.01 (1%), we found that 3,291 unigenes (Supplementary Table 1) were differentially expressed in infected 'Tendral' samples relative to mock-inoculated controls, whereas 2,488 unigenes were differentially expressed in infected TGR-1551 samples. We found that 677 unigenes were deregulated in both genotypes. Samples were clustered based on the expression of deregulated unigenes, showing that they grouped first by days postinoculation and then by infection status (Fig. 3A), specially in 'Tendral'. In the case of TGR-1551, samples clustered depending on whether or not they were infected, probably due to the bigger transcriptomic alterations

consequence of the infection observed in this genotype (Fig. 2A, PCA analysis). Box-plot diagrams showing the fold changes of deregulated unigenes indicated that the amplitude of deregulation was greater in the resistant than the susceptible genotype (Fig. 4).

We next identified functional categories that were over-represented in deregulated unigenes using the Gene Ontology (GO) (Ashburner et al. 2000) vocabulary. The results are summarized in Figure 5 with a set of nonredundant GO categories, whereas a complete list is provided in Supplementary Fig. S1. In TGR-1551, the GO terms "chloroplast thylakoid lumen" and "cellulose and pectin-containing cell wall" were over-represented in deregulated unigenes, whereas "ribosome biogenesis and assembly" and "translation" were under-represented. In contrast, several GO terms related to translation were over-represented among the deregulated unigenes in 'Tendral', whereas chloroplast-related GO terms were under-represented. When the 677 unigenes that were deregulated by WMV infection in both genotypes were used to search statistically significant GO terms, the categories "chromatin assembly" and localization in the "endomembrane system" were identified (data not shown).

We performed additional analysis using viral load as a continuous variable instead of time in the regression model because the kinetics of viral RNA accumulation was different in both melon genotypes and, at 7 dpi, there was a significant difference in virus accumulation (Fig. 1A). In this new analysis, using an FDR of 0.05 (5%), 77 unigenes were found to be differentially expressed in 'Tendral' and 111 in TGR-1551, and only 3 were common to both genotypes. Based on expression data, samples were clustered in this case first by infection

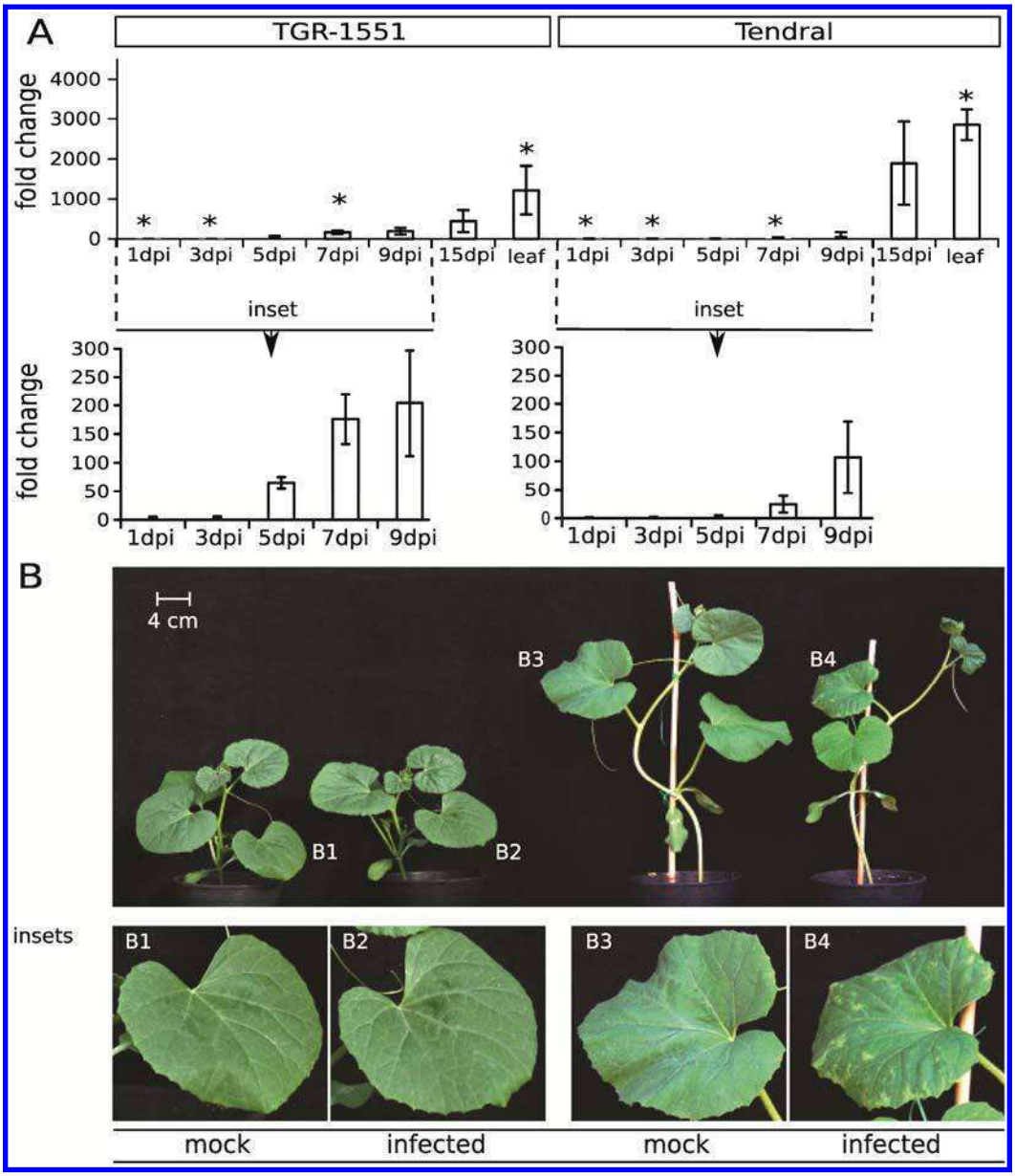

Fig. 1. Viral load and symptoms in melon plants infected with *Watermelon mosaic virus* (WMV). **A,** Pattern of RNA accumulation in 'Tendral' (susceptible) and TGR-1551 (resistant) plants as measured by quantitative polymerase chain reaction. Infected samples at 1 day postinoculation (dpi) were used as calibrators for relative quantification. Samples from inoculated cotyledons were harvested from 1 to 15 dpi, and the systemically infected second true leaf was harvested at 15 dpi. Biological replicates of pooled RNA samples (two in the case of mock-inoculated cotyledons, four in the case of infected cotyledons, and three in the case of leaf samples) were used for each days postinoculation–genotype combination. Asterisks show the samples selected for microarray hybridizations. RNA accumulation in samples at early stages (1 to 9 dpi) is shown in the insets at a different scale. **B**, Melon plants used for this analysis. Second true leaves of healthy and virus-infected plants from each genotype are shown in the insets: B1, mock-inoculated TGR-1551 plant; B2, WMV-infected TGR-1551 plant; B3, mock-inoculated Tendral plant; B4, WMV-infected Tendral plant.

status and then by days postinoculation (Fig. 3B). Interestingly, infected samples at 7 dpi grouped more independently relative to the other samples in both genotypes. No significantly deregulated GO terms were found in the corresponding sets of unigenes. Manual inspection of the unigene annotations identified transcripts encoding proteins located in the endomembrane system and unigenes coding for methyltransferases, enzymes related to fatty acid metabolism (some of them localized in chloroplasts), a copper-binding protein (laccase), peroxidases, a pyruvate kinase, WRKY transcription factors and, glucanases.

Genes differentially expressed in systemically infected leaves.

Deregulated genes in leaves were identified using SAM (Tusher et al. 2001). Much more biological variability was found in 'Tendral' compared with TGR-1551 replicates; therefore, differentially expressed unigenes were identified at different levels of confidence for each genotype. Using an FDR of 1%, 1,886 deregulated unigenes were identified in the resistant genotype, whereas only 121 unigenes were found in the susceptible genotype even with an FDR of 19%, strongly suggesting more profound transcriptome remodeling in the resistant genotype. Box-plot diagrams (Fig. 4B) revealed that, in addition to the number of deregulated unigenes, the amplitude of deregulation was also higher in TRG-1551 leaves. Up to 30 unigenes were deregulated in both genotypes.

The GO categories over-represented in these sets of unigenes are summarized in Figure 6. In 'Tendral', no significant terms were identified among the 22 downregulated unigenes and, among the 99 upregulated genes, the most abundant transcripts were those related to toxin metabolic processes, the response to salicylic acid stimulation, and glutathione transferase activity (Fig. 6). In TGR-1551, GO terms related to biotic stimuli and responses to other organisms were over-represented among the deregulated unigenes, mainly reflecting the accumulation of transcripts involved in pathogen defense (e.g., WRKY and MYB transcription factors, and pathogenesis-related [PR]-like proteins) and stress responses (e.g., peroxidases, lipoxigenases, and heat-shock proteins) (Fig. 6). Cytoskeleton-related unigenes were downregulated in both genotypes, albeit with a

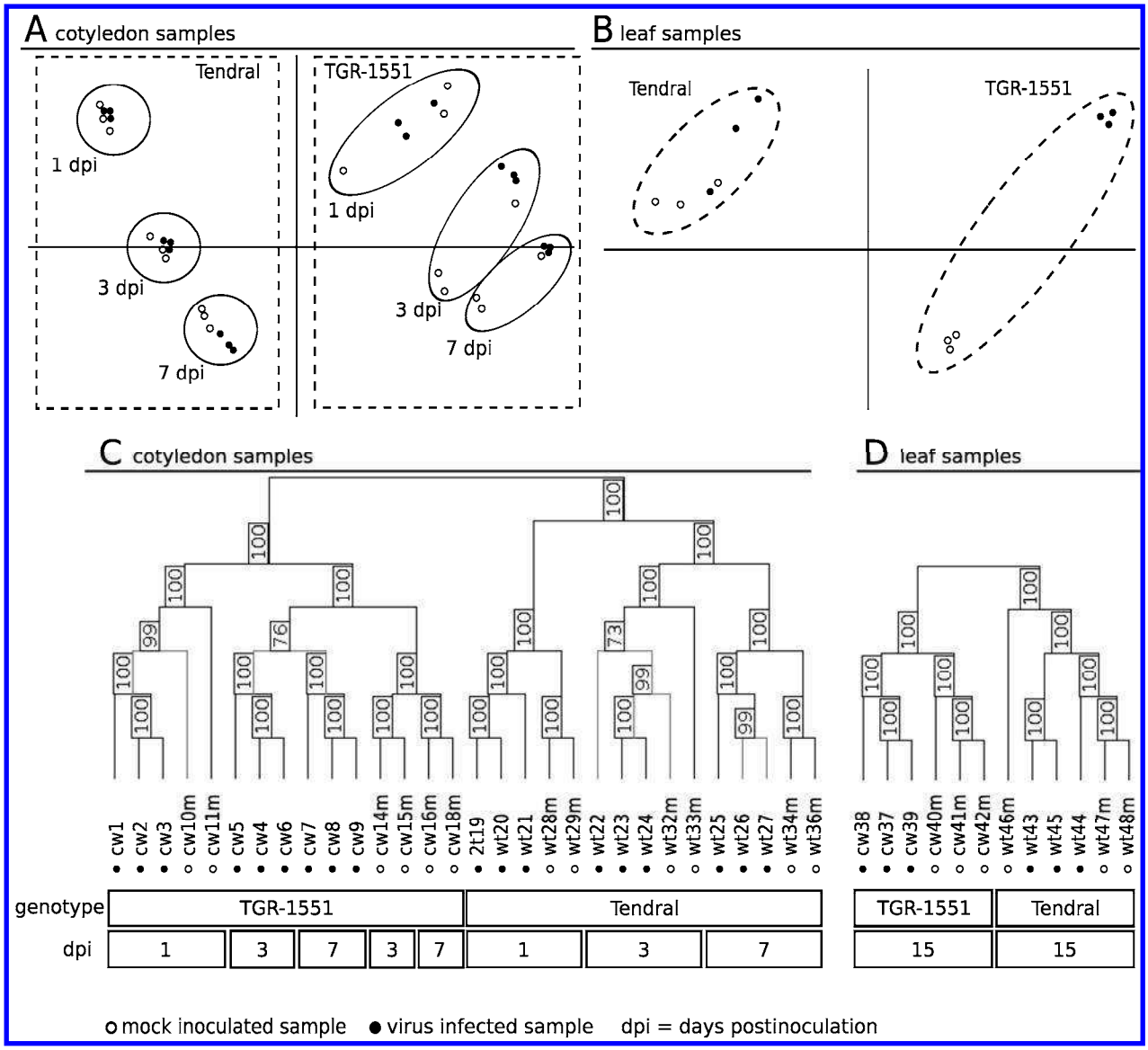

Fig. 2. Analysis of biological variability in microarray samples. **A,** Principal component analysis (PCA) of cotyledon samples at 1, 3, and 7 days postinoculation (dpi) for the TGR-1551 (resistant) and 'Tendral' (susceptible) melon genotypes analyzed after normalization by microarray. Dashed lines: samples grouped by genotype. Black continuous lines: samples grouped by days postinoculation. **B,** PCA for leaf samples. Dashed lines: samples grouped by genotype. **C,** Dendrogram obtained after bootstrapped clustering of cotyledon samples. **D,** Dendrogram obtained after bootstrapped clustering of leaf samples.

greater amplitude in TGR-1551. The exception was unigene cA_15-G12-M13R_c, which is related to microtubule-associated protein RP/EB. This was upregulated strongly in the resistant genotype but downregulated in susceptible plants. Heat-shock proteins were strongly upregulated in TGR-1551 leaves but appeared unaffected in 'Tendral' plants. In contrast, DNAJ-like proteins, which are also involved in protein folding, were downregulated in TGR-1551 leaves. These data provide evidence of a complex resistance response in TGR-1551 leaves following WMV infection.

Expression profiles of defense, stress and endomembrane system genes.

We compared the expression profiles of differentially expressed unigenes in the leaves and cotyledons of both genotypes. Many of the unigenes that were deregulated in leaves were also deregulated in cotyledons, suggesting similar underlying processes in both tissues. For example, unigenes encoding proteins involved in phytohormone biosynthesis and signaling (gibberellins, auxins, and cytokinins) followed this general trend (data not shown). Similarly, more than 150 unigenes re-

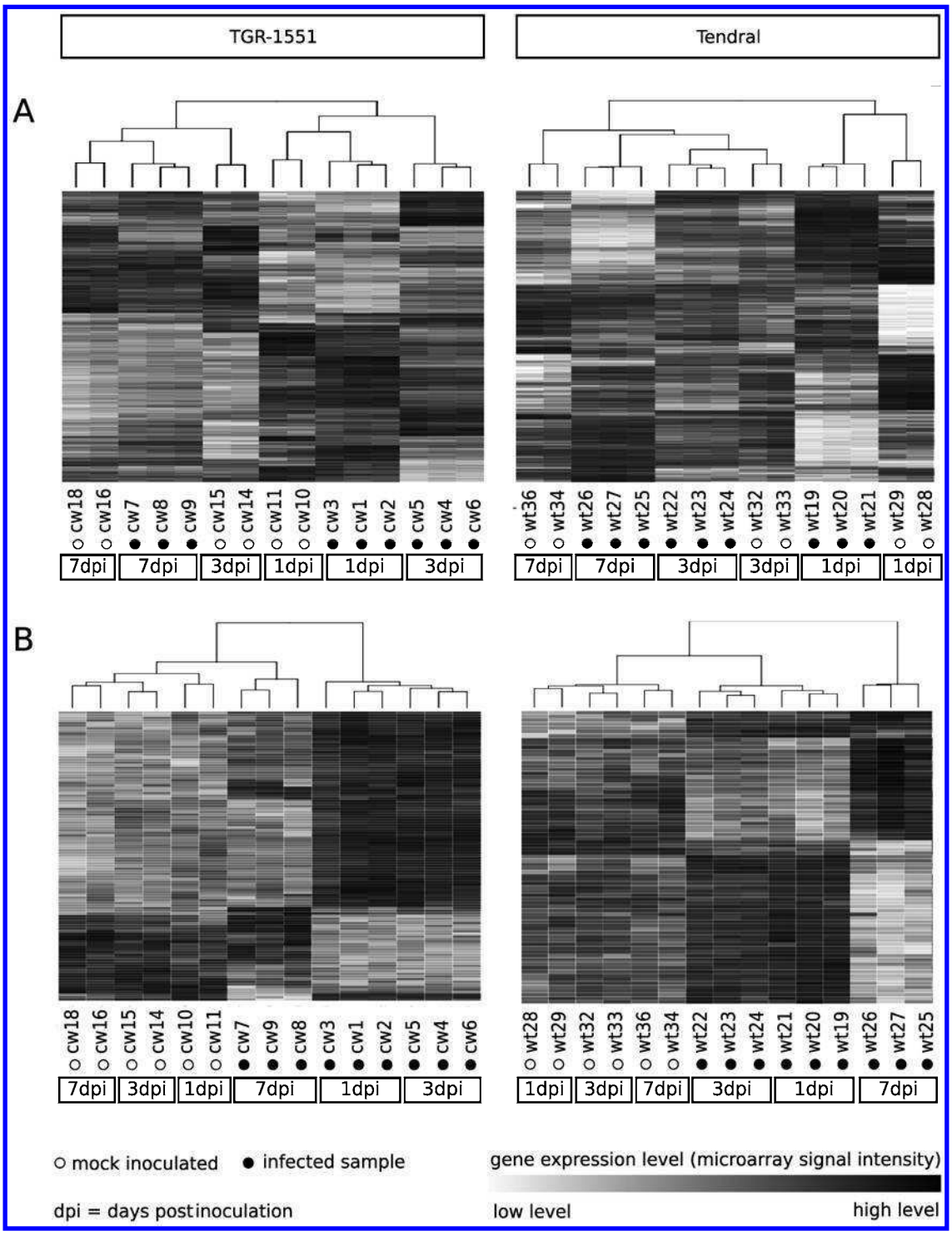

Fig. 3. Cluster analysis of samples based on identified deregulated unigenes. Expression image of significant differentially expressed unigenes identified using the microarray significant profile R package by using **A,** time or **B,** viral load as the continuous variable. Samples used for microarray hybridizations were clustered based on the expression of these deregulated unigenes and the dendrogram is shown for each genotype.

lated to endomembrane system functions were strongly deregulated in TGR-1551 leaves and cotyledons, and deregulated to a lesser extent in the 'Tendral' genotype.

Several transcripts with defense and stress-response functions showed differential accumulation profiles among the four genotype–tissue combinations, although a similar pattern emerged with stronger deregulation in TGR-1551 than 'Tendral'. The expression profiles of selected unigenes are shown in Figure 7, and profiles of sets of unigenes grouped by functional annotation are shown in Supplementary Figure S2. Unigenes in the "response to oxidative stress" category, especially peroxidases, were the most abundant. They were strongly deregulated in TGR-1551 leaves, weakly deregulated in 'Tendral' leaves, and moderately deregulated in the cotyledons of both genotypes. They also showed different expression dynamics, maintaining induction until 7 dpi in TGR-1551 but peaking at 1 dpi and declining thereafter in 'Tendral' plants; for example, unigene cAI_14-A12-M13R_c (Fig. 7). Unigenes annotated with the GO term "response to other organisms" (most of them lipoxigenases) were clearly upregulated in TGR-1551 leaves but were not affected or downregulated in 'Tendral' leaves and cotyledons; for example, unigene cPSI_25-A03-M13R_c (Fig. 7). MYB transcription factors were preferentially upregulated in TGR-1551 leaves and showed stronger deregulation in TGR-1551 cotyledons compared with 'Tendral', in some cases with opposite trends; for example, unigene cAI_15-F09-M13R_c (Fig. 7).

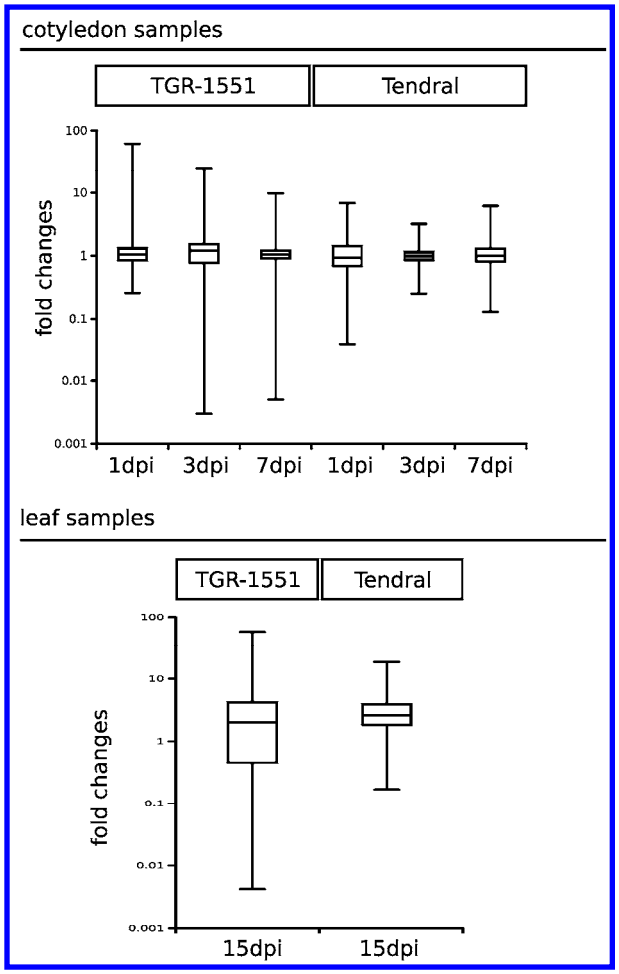

Fig. 4. Broad gene expression trends in samples analyzed by microarray. Gene expression fold changes were calculated for deregulated unigenes identified by microarray analysis and used to construct box plots for each genotype–tissue–days postinoculation (dpi) combination. Results are shown for cotyledon and leaf samples.

Several PR-like proteins were upregulated in TGR-1551 and 'Tendral' leaves, in principle with similar amplitudes but potentially with higher intensities in TGR-1551 due to microarray signal saturation effects (see below and Discussion). Similarly, chitinases, glutathione-S-transferases, and WRKY transcription factors were also deregulated in leaves of both genotypes but different accumulation dynamics were observed in cotyledons, showing stronger deregulation in TGR-1551; for example, unigene cAI_21-H01-M13R_c (Fig. 7). Germin-like proteins annotated with the "apoplast" GO term, dirigent-like proteins, and syntaxins were more strongly upregulated in TGR-1551 leaves than 'Tendral' leaves but also deregulated in cotyledons. Several UDP-glucosyltransferases were upregulated in TGR-1551 leaves but not deregulated in 'Tendral' leaves. In contrast, unigenes encoding phenylalanine ammonia lyase were upregulated in 'Tendral' leaves but not deregulated in TRG-1551 leaves. Other transcripts, such as those annotated as nucleotide-binding site leucine-rich repeat proteins, showed weak deregulation in both tissues of both genotypes. These data support the induction of a complex resistance response in TGR-1551 plants following WMV infection.

Microarray data validation by real-time qPCR.

We validated the microarray expression data by using real-time qPCR to measure the accumulation of five melon transcripts in the samples used for microarray hybridizations. We chose transcripts encoding HSP17.6, two PR proteins, a calmodulin-binding protein, and a chitinase A, all potentially involved in pathogenesis (Table 1). In total, 210 expression values (150 from cotyledons and 60 from leaves) from both microarray and qPCR experiments were analyzed, the fold changes were calculated, and expression patterns were compared (Supplementary Fig. S3). In cotyledon samples, the qPCR data for four of the transcripts matched the differential trends between the genotypes observed in the microarray experiments. The exception was unigene MU10940 at 3 dpi in TGR-1551, which showed lower variation than was apparent from the microarray data. In leaf samples, different trends were observed for each genotype, with 'Tendral' samples showing good correlation in tendency and amplitude but TGR-1551 samples showing a correlation in tendency but not in amplitude (qPCR indicated higher fold changes than the microarray data). However, the expression profiles of the five transcripts were mostly coincident, with Pearson correlation coefficients ranging from 0.75 to 0.96 (Table 1).

DISCUSSION

Microarray analysis has been widely used to compare the transcriptomes of virus-infected plants and healthy controls (Agudelo-Romero et al. 2008; Babu et al. 2008; Catoni et al. 2009; Dardick 2007; Espinoza et al. 2007; Gandía et al. 2007; Golem and Culver 2003; Pompe-Novak et al. 2005; Satoh et al. 2009; Senthil et al. 2005; Smith et al. 2004; Whitham et al. 2003). However, few of these studies have focused on incompatible plant–virus interactions with the intention of gaining information about resistance mechanisms (Gammelgård 2007; Marathe et al. 2004; Shi et al. 2005). We used microarrays to study TGR-1551 resistance to WMV by comparing transcriptome responses in this accession and a susceptible variety.

Differentially expressed unigenes.

Over 3,000 differentially expressed unigenes were identified in cotyledons when time was used as a continuous variable in the MaSigPro program. Clustering based on expression data showed that the samples grouped preferentially by days postinoculation for every genotype, suggesting that the large

number of deregulated unigenes represented transcriptomic changes over time rather than in response to infection. We attribute this result to the slow progression of the WMV infection, characteristic of *Potyvirus* spp., as noted by the low level of WMV RNA accumulation until 9 dpi (Fig. 1). This phenomenon was also genotype dependent, because larger transcriptome remodeling was found at early stages in cotyledons of the resistant genotype. However, for both genotypes, a manual inspection of the expression patterns after clustering (data not shown) revealed that many of the genes follow similar trends in healthy and infected samples. Therefore, a significant number of the deregulated unigenes expressed in cotyledons may represent false positives (i.e., their modulation may be incorrectly attributed to the infection). This may partially reflect the low biological dispersion observed among replicates, combined with the relatively small variations in expression observed in cotyledons.

In contrast, when the analysis was carried out using viral load as a continuous variable, only approximately 100 deregulated unigenes were identified for each genotype, in agreement with the PCA data. Samples clustered first by infection status and then by time, indicating that the new set of unigenes represented transcriptomic changes due to infection rather than the experimental time course. However, we believe that these experiments were complementary and that both have advantages and drawbacks. For example, many infection-related genes may also be modulated over time, making them impossible to identify when using viral load as continuous variable. The time course analysis could, for example, identify genes whose expression profile changes during the course of infection (e.g., in response to the increasing viral titer). Examples of such unigenes included type III homocysteine methyltranferases, laccases, enoyl-ACP reductases, calmodulin-binding proteins, peroxidases, and auxin response factors, many of them already known to be involved in plant–pathogen interactions (Abdel-Ghany and Pilon 2008; Benschop et al. 2007; Chandra-Shekara et al. 2007; Raffaele et al. 2008).

Mechanism of TGR-1551 resistance to WMV.

TGR-1551 resistance to WMV may involve the restriction of virus movement (Diaz-Pendon et al. 2005), which would be compatible with both active and passive resistance mechanisms (Fraser 1992). If resistance were passive, as the recessive genetics would suggest, one should expect no activation of resistance responses in relation to appropriate controls. For example, functional genomics has been used to analyze potato resistance to *Potato virus A (*genus *Potyvirus,* family *Potyviridae*), which involves the inhibition of virus translocation from infected leaves. In this case, gene induction in resistant plants was only slightly greater than in susceptible ones (Gammelgård 2007). In contrast, our microarray results showed robust and distinct transcriptome remodeling in infected TGR-1551 plants, especially in leaf samples, involving many pathogen response transcripts. This indicated that a defense response associated with resistance is mounted in infected TGR-1551 plants.

How can this active resistance mechanism be reconciled with the recessive genetics of the resistance trait? One possible explanation is the absence or reduced expression of host factors that counteract resistance responses. Although such a mechanism has not yet been observed in virus infections, several cases can be cited for other pathogens. For example, recessive alleles generated by mutagenesis at the barley *MLO* locus are responsible for wide-spectrum resistance to fungal pathogens (Büschges et al. 1997). There are 15 members of this protein family in *Arabidopsis* and, among seven homologous melon unigenes represented on our microarray, two were found to be deregulated. One was downregulated in 'Tendral' cotyledons at 1 dpi but was not deregulated in TGR-1551 cotyledons (data not shown), suggesting that 'Tendral' cotyledons

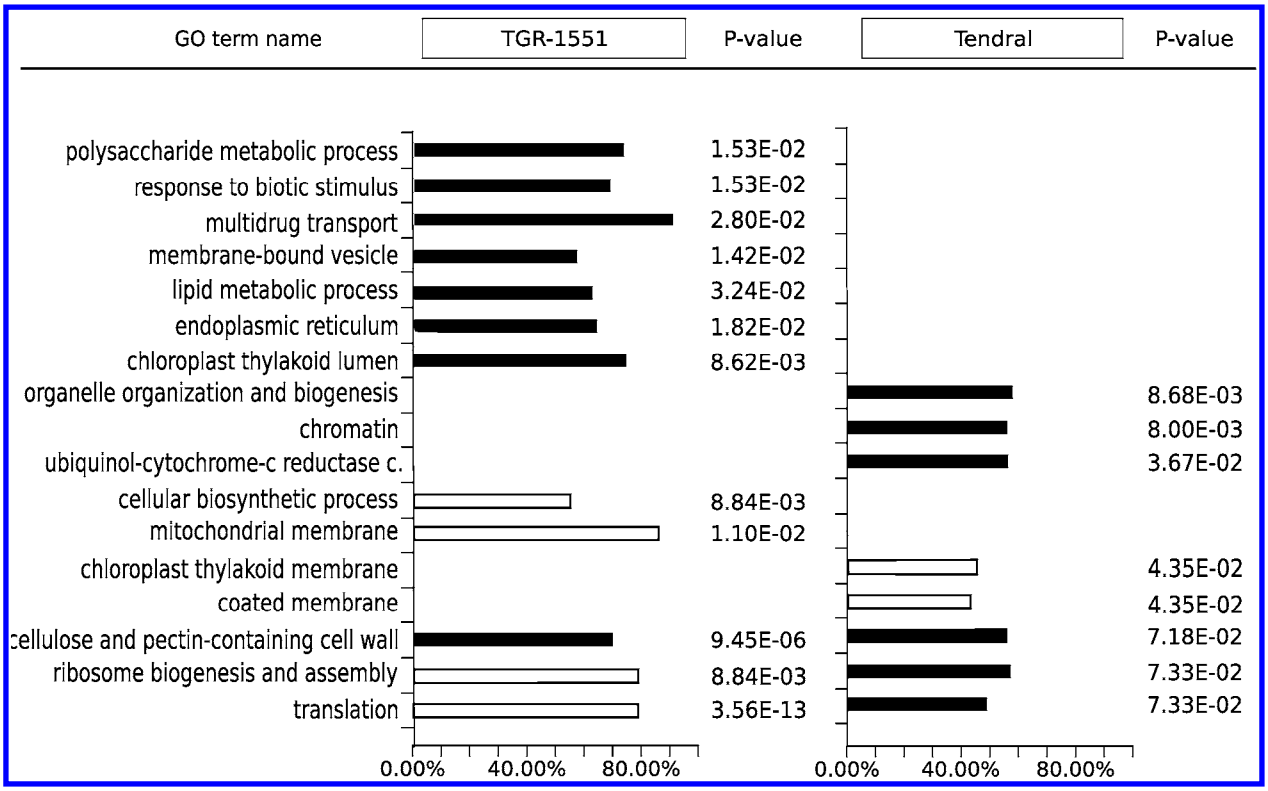

Fig. 5. Significant Gene 0ntology (GO) categories among the deregulated unigenes in cotyledons. Differentially expressed unigenes identified by the microarray analysis of cotyledon samples were used to analyze statistically significant GO terms, over-represented (black) and under-represented (white), for each genotype. Percentage of deregulated unigenes from the total number of unigenes included in each GO category is indicated on the horizontal axis. A selection of nonredundant categories is shown here.

may attempt a defense response that is not initiated in TGR-1551 plants. The other was downregulated in TGR-1551 leaves but not deregulated in 'Tendral' leaves, and this is an interesting candidate for further functional analysis. Other examples include *Arabidopsis* mutants with recessive alleles at the *SSI2* (stearoyl-ACP desaturase) locus, which allow the constitutive accumulation of the transcript encoding PR-1 as well as salicylic acid, conferring resistance against bacteria, fungi, and *Cucumber mosaic virus* (Sekine et al. 2004). A melon transcript similar to a stearoyl-ACP desaturase was identified but its expression was strongly repressed at 7 dpi in 'Tendral' plants and was not deregulated in TGR-1551 plants.

Genetic resistance to plant viruses often involves a small and highly conserved collection of genes (Kang et al. 2005; Truniger and Aranda 2009) (e.g., the eIF4E and eIF4G translational initiation factor genes involved in recessive resistance, especially to *Potyvirus* spp.) (Robaglia and Caranta 2006; Truniger and Aranda, 2009). Many cases of eIF4E-mediated resistance reflect a lack of virus multiplication at the cellular level. In some cases, however, eIF4E-mediated resistance still allows the systemic accumulation of virus particles, albeit to a lesser extent and without symptoms (Nicaise et al. 2003; Revers et al. 1997). This is similar to the situation in TGR-1551 plants infected with WMV but we did not identify any differentially expressed transcripts homologous to *Arabidopsis* eIF4E or eIF4G when comparing 'Tendral' and TGR-1551 plants (data not shown). Furthermore, sequencing eIF4E cDNAs and gene exons has not shown any difference between TGR-1551 and susceptible genotypes (Nieto et al. 2007). Even so, this hypothesis should not be completely ruled out because not all the translation initiation factors have been sequenced and characterized in TGR-1551, and there may be differences between transcript levels, protein levels, and protein activity that make mRNA profiling an unreliable indicator of the role of these proteins in resistance.

The resistance mechanism could also involve small RNAs. Recently, we screened the melon small RNome by pyrosequencing, and noted the differential accumulation of miR168,

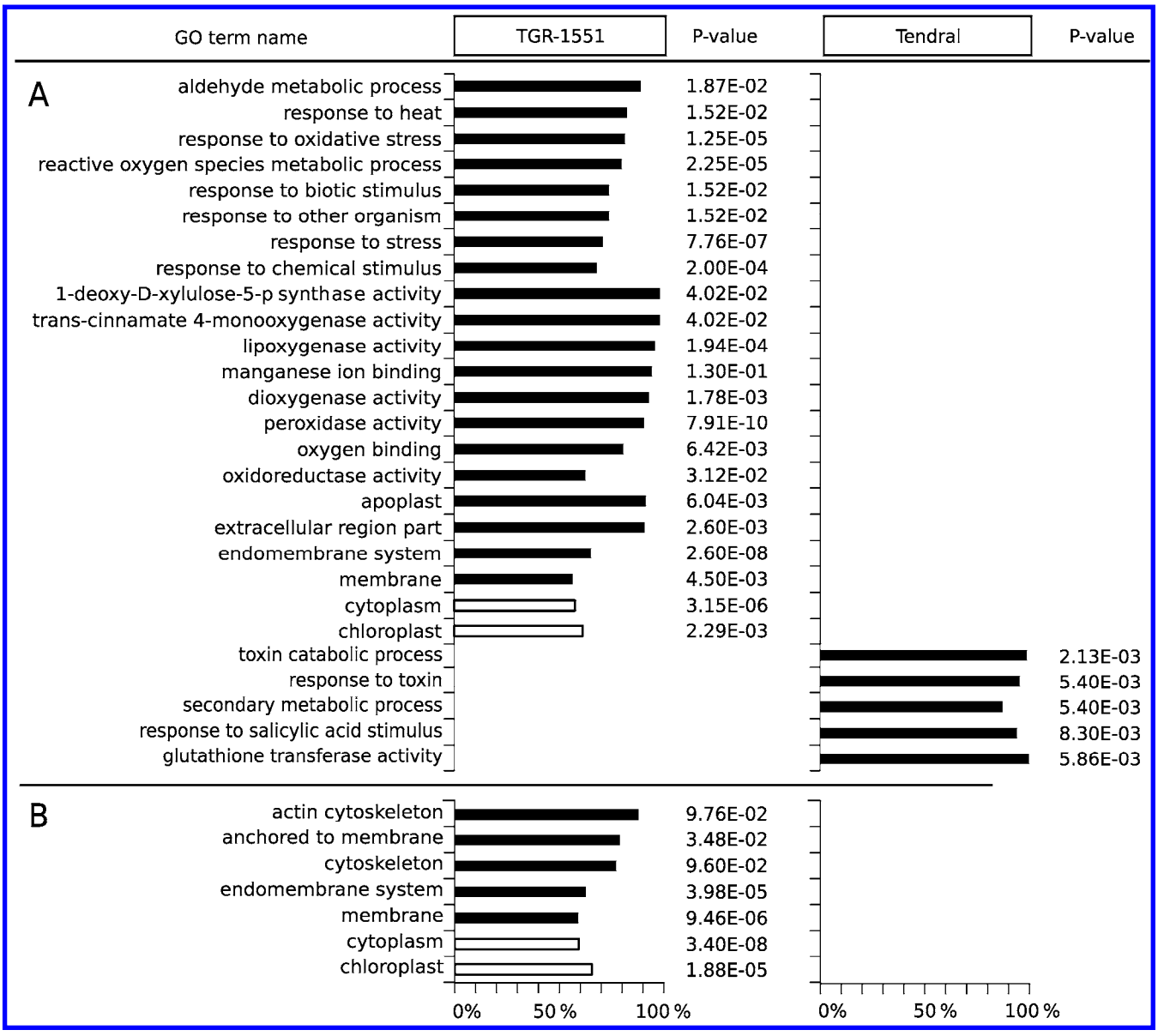

Fig. 6. Significant gene ontology (GO) categories among the deregulated unigenes in leaves. Differentially expressed unigenes identified by the microarray analysis of leaf samples were used to analyze statistically significant GO terms, over-represented (black) and under-represented (white), for each genotype. Percentage of deregulated unigenes from the total number of unigenes included in each GO category is indicated on the horizontal axis. **A,** Significant GO terms in upregulated unigenes and **B,** significant GO terms in downregulated unigenes.

which was expressed at high levels in TGR-1551 and at low levels in 'Tendral' (Gonzalez-Ibeas et al. 2011). This microRNA regulates the transcript for ARGONAUTE1 (AGO1), the catalytic subunit of the RNA-induced silencing complex responsible for post-transcriptional gene silencing (Vaucheret et al. 2006). Melon unigenes representing AGO proteins also showed differential trends between TGR-1551 and 'Tendral' in our microarray experiments, two in cotyledons and one in leaves. Interestingly, some melon unigenes annotated as transposons also showed different degrees of deregulation between the genotypes (i.e., strongly deregulated in TGR-1551 and not deregulated at all in 'Tendral'). Stress (including stress caused by pathogens) can modulate the transcriptional activity of transposons, and RNA interference can interfere with this pathway to activate transposable elements under non-stress conditions (Madlung and Comai 2004). The accumulation of miR168, the expression of the melon *AGO* transcripts, and the expression of transposon unigenes may suggest the potential involvement of the silencing machinery in TGR-1551 resistance to WMV, although further work is required to investigate this hypothesis.

Validation of the microarray data showed a strong correlation between the qPCR and microarray datasets although, in TGR-1551 leaf samples, we noted that there was good correlation in the gene expression tendencies but not the amplitudes. Expression profiles often have a larger amplitude when detected by qPCR compared with microarray data (Dardick 2007; García-Marcos et al. 2009), and signal saturation effects for strongly expressed transcripts have also been observed when using microarrays (Lee 2004). The strongly expressed unigenes in TGR-1551 may reach this saturation limit, resulting in underestimated expression levels and therefore suggesting that transcriptomic variations in TGR-1551 leaves are greater than indicated by microarray hybridization, supporting the hypothesis that large-scale transcriptomic remodeling occurs in infected TGR-1551 plants. In addition to pathogen response transcripts, this robust transcriptome remodeling affected multiple metabolic processes and molecular functions. One interesting case was the deregulation of transcripts coding for ribosomal proteins. Ribosomes are key elements in the synthesis of proteins and there is evidence of different

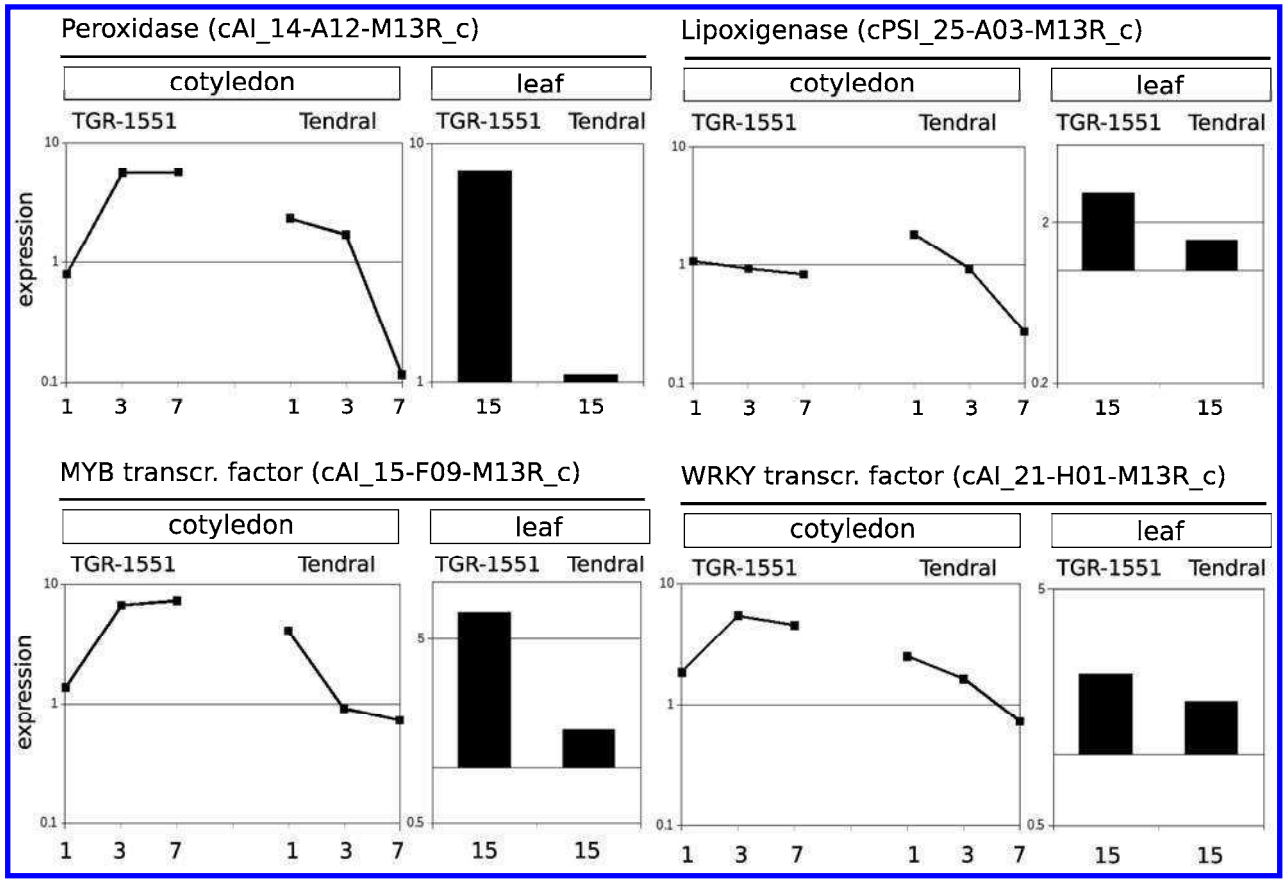

Fig. 7. Gene expression patterns of deregulated melon transcripts. For cotyledon samples, results are shown at 1, 3, and 7 days postinoculation (dpi) (*x* axis). For leaf samples, results are shown at 15 dpi (*x* axis). Gene expression fold changes in infected samples relative to mock-inoculated controls are shown on the *y* axis. Each unigene is named in parenthesis.

Table 1. Selected melon transcripts for microarray validation data by quantitative polymerase chain reaction

Melon unigene[a]	*Arabidopsis* gene	Functional annotation	Pearson correlation (cotyledon)[b]	Pearson correlation (leaf)[b]
cCL5001Contig1	AT1G53540	17.6-kDa class I small heat shock protein (HSP17.6C-CI)	0.91	0.87
cCI_58-B08-M13R_c	AT3G04720	Hevein-like protein (HEL); wound-induced protein WIN2 precursor	0.96	0.89
cFR15P6_c	AT5G24090	Acidic endochitinase (CHIB1)	0.82	0.80
MU10940	AT2G14610	Pathogenesis-related protein (PR)	0.87	0.75
cA_31-B03-M13R_c	AT3G13600	Calmodulin-binding family protein	0.84	0.80

[a] *Arabidopsis* gene used for melon unigene annotation by sequence similarity.
[b] Pearson coefficient correlation between microarray data and quantitative polymerase chain reaction results.

ways to recruit them by viruses (Carroll et al. 2008; Doudna and Rath 2002). Recently, it has been described that ribosomal protein mRNAs show increased accumulation in *Potyvirus* infection of *Arabidopsis*, their induction is coordinated in response to infection, and several of them have been shown as cellular host factors required by some viruses for infection of *Nicotiana benthamiana* (Yang et al. 2007, 2009). Twenty-nine melon unigenes annotated as ribosomal proteins were found notoriously deregulated in leaves of TGR-1551 compared with 'Tendral' over the rest of ribosomal protein transcripts, which remained unaltered, suggesting specific deregulation and, perhaps, a potential role in the virus infection process. The same observation was extensive to other gene categories such as plant hormone metabolism, endoplasmic reticulum localization, or cell wall maintenance, revealing a deep adaptation of the plant physiology to the infection and highlighting microarrays, as broadly reported in many works, as powerful tools for identifying specific genetic elements involved in the process under study.

MATERIALS AND METHODS

Plant material, viral isolate, and virus inoculation.

Melon (*Cucumis melo* L.) accession TGR-1551 was originally collected in Zimbawe and shows resistance to WMV (Diaz-Pendon et al. 2005; Soria et al. 2003). TGR-1551 seed were kindly provided by Dr. Gómez-Guillamón (Estación Experimental "La Mayora"-CSIC, Málaga, Spain). 'Tendral' melon (Semillas Fitó, Barcelona, Spain) was used as the susceptible control. Seed were germinated in petri dishes for 48 h at 25°C, and then sown in 0.5-liter pots maintained in growth chambers (MLR-351H from Sanyo) with a 16-h photoperiod and 25 and 18°C day and night temperatures, respectively, for 3 weeks. Viral isolate WMV-M116 (Diaz-Pendon et al. 2005) was kindly provided by Dr. Moriones (Estación Experimental "La Mayora"-CSIC). Mechanical inoculations were carried out using standard procedures after the dehydrated viral inoculum was revived by mechanical inoculation of fully expanded squash cotyledons. Systemically infected squash leaves were harvested 15 dpi and used as the inoculum for melon plants. The leaves were ground in a sterile mortar in the presence of inoculation buffer (0.2 M phosphate [pH 8.0], 0.1% [vol/vol] β-mercaptoethanol, and active charcoal at 0.03 g/ml), mixed with 0.037-mm Carborundum particles, and dusted onto 7-day-old melon cotyledons. Control noninfected melon cotyledons were rubbed using the inoculation buffer alone (mock-inoculated controls).

Time course experiment, RNA extractions, and microarray hybridizations.

For each genotype, 60 melon seedlings were inoculated with WMV-M116 and another 60 were mock inoculated. Cotyledons were harvested from 10 plants at 1, 3, 5, 7, 9, and 15 dpi. Harvested plants were removed from the assay after each harvest. At 15 dpi, the systemically infected second true leaf was also harvested. By this time, 'Tendral' second leaves showed evident mosaic and malformation symptoms, resembling those typically induced by WMV, whereas TGR-1551 plants were symptomless (Fig. 1). Samples were independently frozen in liquid N_2 and stored at –80°C. RNA extracts were prepared using Tri-Reagent (Sigma-Aldrich, St. Louis) following the manufacturer's instructions. WMV infection was checked by dot-blot hybridization (Kassem et al. 2007) using 1 μg of RNA extract. To reduce variability, each biological replicate was prepared by mixing the RNA extracts from two or four mock- or WMV-inoculated cotyledons, respectively, or from three melon leaves. To eliminate traces of genomic DNA, total RNA was incubated with DNAse I (New England Biolabs, London) for 10 min at 37°C. The reaction volume was adjusted to 100 μl and the aqueous phase extracted with phenol/chloroform/isoamyl alcohol (125:24:1). Total RNA was precipitated with 10% (vol/vol) NaCl 3M and 2.5 volumes of absolute ethanol by centrifugation (12,000 × g, 20 min, 4°C). The quantity and quality of RNA were determined using a ND-1000 spectrophotometer (Nano Drop Technologies, Wilmington, DE, U.S.A.) and a Bioanalyzer (Agilent Technologies, Palo Alto, CA, U.S.A.). All samples were used for viral load quantification, and samples corresponding to 1, 3, and 7 dpi were selected for microarray analysis and were sent for further processing at the NimbleGen microarray hybridization service (Roche NimbleGen Iceland LLC, Reykjavik, Iceland). Processing consisted of cDNA synthesis, Cy3 cDNA labeling, hybridization, scanning, and image reading. The data discussed in this publication have been deposited in the National Center for Biotechnology Information's GEO (Barrett et al. 2011) and are accessible through GEO Series accession number GSE30111.

Real-time reverse-transcription qPCR.

Real-time qPCR was carried out using an AB 7500 System (Applied Biosystems, Foster City, CA, U.S.A.), with Power SYBR green dye (Applied Biosystems) and ROX as a passive reference. *CYCLOPHILIN* mRNA was used as the endogenous control (Gonzalez-Ibeas et al. 2007), ΔΔ-cycle threshold was the algorithm for relative quantification, and three technical replicates were used for statistical analysis. Melting curve analysis at the reaction end-point and no-template controls were used to ensure product-specific amplification and to avoid primer-dimer quantification. A control reverse transcription without the enzyme was carried out to evaluate genomic DNA contamination. Primers to quantify the melon transcripts were designed using Primer Express (Applied Biosystems) or Primer3 (Rozen and Skaletsky 2000) from EST sequences in the Melogen database. Primers to quantify WMV-M116 RNA were designed using the virus sequence from GenBank (accession number AF551334).

Data analysis.

Data produced by the NimbleGen service were normalized using the RMA algorithm within oligo (vs. 1.8.2) (Carvalho et al. 2007) written in R (vs. 2.9.1) (R Project for Statistical Computing website). Density histograms and box-plot diagrams were generated using the same package and used to confirm that the data were efficiently normalized and the technical variability was acceptable for downstream analysis (Supplementary Fig. S4). Differentially expressed genes were identified using MaSigPro (v. 1.16.0) (Conesa et al. 2006) written in R, and the SAM module (Tusher et al. 2001) of the Multiexperiment viewer (MeV) (v. 4.4.1) program (Saeed et al. 2006). Clustering analysis was carried out using the MeV module Hierarchical Clustering Support Trees (ST) (Eisen et al. 1998). Biological variability was estimated using the MeV PCA module (Raychaudhuri et al. 2000). Over-represented GO (Ashburner et al. 2000) terms among the differentially expressed genes were identified using the *FatiGO* module (Al-Shahrour et al. 2007) in the Babelomics (v. 3) suite (Al-Shahrour et al. 2008). GO terms with an adjusted P value < 0.1 for multiple comparisons were considered statistically significant.

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Publicación II.

Título: Analysis of the melon (*Cucumis melo*) small RNAome by high-throughput pyrosequencing

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Aportación del doctorando: Cultivo de las plantas de melón e inoculación del virus en las muestras infectadas con el virus del mosaico de la sandía. Elaboración de las 12 genotecas de pequeños RNAs bajo tutela de César LLave Correas (CIB, Madrid). Análisis bioinformático de los resultados de secuenciación bajo tutela de Jose Blanca Postigo (COMAV, Valencia). Diseño experimental y escritura del manuscrito bajo tutela de Miguel A. Aranda.

BMC Genomics

RESEARCH ARTICLE Open Access

Analysis of the melon (*Cucumis melo*) small RNAome by high-throughput pyrosequencing

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Abstract

Background: Melon (*Cucumis melo* L.) is a commercially important fruit crop that is cultivated worldwide. The melon research community has recently benefited from the determination of a complete draft genome sequence and the development of associated genomic tools, which have allowed us to focus on small RNAs (sRNAs). These are short, non-coding RNAs 21-24 nucleotides in length with diverse physiological roles. In plants, they regulate gene expression and heterochromatin assembly, and control protection against virus infection. Much remains to be learned about the role of sRNAs in melon.

Results: We constructed 10 sRNA libraries from two stages of developing ovaries, fruits and photosynthetic cotyledons infected with viruses, and carried out high-throughput pyrosequencing. We catalogued and analysed the melon sRNAs, resulting in the identification of 26 known miRNA families (many conserved with other species), the prediction of 84 melon-specific miRNA candidates, the identification of *trans*-acting siRNAs, and the identification of chloroplast, mitochondrion and transposon-derived sRNAs. *In silico* analysis revealed more than 400 potential targets for the conserved and novel miRNAs.

Conclusion: We have discovered and analysed a large number of conserved and melon-specific sRNAs, including miRNAs and their potential target genes. This provides insight into the composition and function of the melon small RNAome, and paves the way towards an understanding of sRNA-mediated processes that regulate melon fruit development and melon-virus interactions.

Background

Melon (*Cucumis melo* L., family *Cucurbitaceae*) is an important horticultural species cultivated in temperate, subtropical and tropical regions worldwide, with Spain being the largest producer in Europe and fifth in the world [1]. The melon genome has 12 chromosomes and is thought to contain 450-500 Mb of DNA, which is 3-4 times more than Arabidopsis [2]. Melon is a useful model for the analysis of fruit traits because of the vast morphological, physiological and biochemical diversity within the species, which can be exploited to dissect the biological processes controlling color, flavor and texture and how these properties arise during fruit development [3,4].

Despite the importance of melon, not much was available in the way of genomic sequence information prior to the establishment of a functional genomics consortium in 2004, which developed a range of tools and accumulated more than 33,000 expressed sequence tags (ESTs) and ~17,000 tentative consensus sequences (unigenes) [5]. This EST collection has been expanded recently with the addition of 94,000 new ESTs from full-length enriched cDNA and standard cDNA libraries from various melon tissues and cultivars in the framework of the International Cucurbit Genome Initiative [6]. These ESTs as well as other resources are now accessible in a public database [7]. The unigene sequences have also been used to construct an oligonucleotide microarray, which has been applied in the analysis of fruit quality traits, ovary development and pathogen resistance [8]. In addition, a melon sequencing consortium has recently produced a high-quality draft of the melon genome (unpublished data). Although these

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resources provided significant advances in the analysis of melon gene expression, the small RNA (sRNAs) component of the melon transcriptome has not been studied in detail. These important molecules have been studied in other crop species and have been shown to fulfill a number of critical regulatory roles [9-12].

sRNAs are short, non-coding RNAs 21-24 nucleotides (nt) in length which are found in protists, fungi, plants and animals [13]. In plants, their roles include maintenance of genome stability, initiation of heterochromatin assembly, post-transcriptional regulation of gene expression and protection against viruses using an RNA-based immune system. The most abundant and best-characterised sRNAs include microRNAs (miRNAs) and small interfering RNAs (siRNAs). miRNAs are widely studied because of their regulatory activity, particularly in development, pathogen resistance and stress responses [13]. miRNAs are cleaved from stem-loop precursor molecules that derive from single stranded non-coding transcripts. miRNAs regulate protein-coding genes post-transcriptionally by mediating RNA cleavage or translational repression. Unlike miRNAs, siRNAs are generated from double-stranded RNA precursors and function on cognate RNA or DNA molecules by instigating degradation or promoting RNA-directed DNA methylation, respectively. cis-acting siRNAs (ca-siRNAs) arise from and target endogenous loci such as transposons and DNA repeats to direct cytosine methylation and chromatin modifications [14]. Natural antisense-transcript siRNAs (nat-siRNAs), which derive from pairs of natural-antisense transcripts, guide the cleavage of one of the two parent transcripts, leading to the production of a series of secondary 21-nt siRNAs of unclear function [15,16]. Finally, trans-acting siRNAs (ta-siRNAs) derived from *TAS* genes, which transcribe long primary non-coding RNAs as precursors for ta-siRNA biogenesis. TAS primary RNAs are cleaved by specific miRNAs and are sequentially processed into 21-nt ta-siRNAs starting from the miRNA-cleaved end, to generate clusters of phased siRNAs [17,18]. In addition to endogenous sRNAs, exogenous siRNAs from virus genomes can be detected in virus-infected plants as a part of the RNA-based immune system [19].

RNA viruses that infect melon are responsible for significant yield losses as well as poor fruit quality [20,21], particularly the widespread *Watermelon mosaic virus* (WMV, genus *Potyvirus*, family *Potyviridae*) [22,23]. Recently, a collection of accessions representing cultivated melon and its wild relatives was screened to identify sources of resistance to mosaic-inducing viruses [24]. TGR-1551 was identified as a resistant accession based on the lower WMV titer compared to susceptible genotypes (e.g. melon cv. Tendral) and the absence or mildness of the mosaic symptoms normally observed in systemically infected leaves [25]. *Melon necrotic spot virus* (MNSV, genus *Carmovirus*, family *Tombusviridae*), although less economically important, may also cause yield losses, and epidemic outbreaks have been reported worldwide [26,23]. In melon, resistance to MNSV is controlled by the single recessive gene *nsv*, which encodes eukaryotic translation initiation factor 4E (Cm-eIF4E) [27]. This resistance is effective against all MNSV strains (e.g. MNSV-Malfa5) except MNSV-264 [28]. Studies of chimeric viruses have shown that the MNSV 3' untranslated region (3'-UTR) contains the resistance-breaking determinant of MNSV-264, and that it functions as a cap-independent translational enhancer [29,30].

We constructed 10 sRNA libraries from a range of healthy and virus-infected melon tissues, and we sequenced a set of endogenous and exogenous sRNAs using the pyrosequencing-based 454 technology from Roche [31]. To gain insights into the role of sRNAs on key aspects of fruit development, maturation and pathogen defense, samples from two stages of the developing ovary, fruits 15 and 45 days after pollination, and photosynthetic cotyledons from resistant and susceptible melon accessions infected with WMV and MNSV were analysed. In a previous study, we reported the profile of virus-derived sRNAs (viRNAs) from cotyledon samples [32]. Here we report a catalog of endogenous melon sRNAs, including miRNAs from known families and new candidate miRNAs potentially unique to melon, focusing on the number of sequence reads as a reflection of their expression profiles. Potential targets for these miRNAs in the melon transcriptome were identified.

Results

cDNA libraries and sequencing of small RNAs

We used high throughput sequencing data to analyze the composition of the small RNA transcriptome (sRNAome) of melon and compare the results to data in publicly-available RNA and genomic databases. Ten sRNA libraries were constructed from total RNA extracted from fruits, ovaries and healthy and virus-infected melon cotyledons (Table 1). PCR amplification products corresponding to each library were pooled in equal amounts and sequences were obtained by multiplexed high-throughput pyrosequencing (Roche 454). This produced 447,180 raw sequences, each ~100 bases in length, 432,743 of which had a complete 3' adaptor in the correct position. Based on these data, we estimated a sequencing error rate of 3.7%. After removing reads where one or the two adaptors could not be identified, 398,450 useful sequences with 3' and 5' adaptors were selected. Only 44 sequences comprising ligated adaptors without an insert were identified. Although we pooled similar amounts of PCR products from each

Table 1 Description of small RNA libraries from different melon tissues

Library	Cultivar/accession	Tissue	Physiological condition	Virus[a]	Reads	Unique sequences
Wtm	cv. Tendral	Cotyledon	Mock-inoculated	–	33123	15624
Wt	cv. Tendral	Cotyledon	Virus-infected	WMV-M116	35860	12840
Cwm	accession TGR-1551	Cotyledon	Mock-inoculated	–	41039	21122
Cw	accession TGR-1551	Cotyledon	Virus-infected	WMV-M116	36330	24100
15d	cv. Piel de Sapo	Fruit	Healthy, 15 days after pollination	–	21662	14620
45d	cv. Piel de Sapo	Fruit	Healthy, 45 days after pollination	–	9942	8167
c1	cv. Piel de Sapo	Ovary	Healthy	–	18764	15269
c5	cv. Piel de Sapo	Ovary	Healthy	–	14529	12608
Ta5	cv. Tendral	Cotyledon	Virus-infected	MNSV-alfa5	43170	22869
3'T	cv. Tendral	Cotyledon	Virus-infected	MNSV (chimeric)	56425	56425

[a]WMV = *Watermelon mosaic virus*; MNSV (alfa5) = *Melon necrotic spot virus*, alfa5 isolate; MNSV (chimeric) = *Melon necrotic spot virus*, alfa5 isolate with 3' UTR from 264 isolate

library, different numbers of sequences were obtained according to the 5' adaptor sequence barcode (Table 1). For instance, the fruit and ovary libraries (15d, 45d, c1 and c5) were poorly represented providing a collection of fewer than one third of the number of sequences from the other six libraries. A set of 186,698 non-redundant sRNA sequences was generated for downstream analysis. The representation of sequences with different lengths in the redundant and non-redundant sRNAs datasets is shown in Figure 1. The most abundant sequences were 21, 24, 20 and 22 nts. A few sequences shorter than 20 nt were also retrieved, and these probably represent cloning artifacts and/or degradation products. Sequences > 30 nt in length in our dataset predominantly represented combinations of other melon sRNAs identified in our work. Detailed data are provided in Additional file 1.

Identification of known miRNAs

In order to identify known miRNAs, the melon sRNA data set was used as a BLAST query against the Arabidopsis small RNA database (ASRP) [33] and the microRNA database (miRbase) [34]. We identified 46 melon unique sequences corresponding to 26 miRNA families. Thirty nine sequences were identical to known miRNAs from other plant species, while 7 additional species were sequence variants highly conserved (up to two mismatches allowed). In order to clearly identify each melon sequence, melon miRNAs were named according to the homologous reference miRNA from each database (Table 2). For each reference miRNA, we found that ~3% of the corresponding melon sequences differed at one or two sites with mismatches distributed randomly along the sequence, so these were considered sequencing errors. Only specific sequence variants that represented more than 3% of the total population for each reference miRNA were considered biologically relevant. We identified only two non-conserved miRNAs, corresponding to ath-miR2111a from Arabidopsis and peu-miR2910 from *Populus euphratica*, respectively (Table 2). The largest diversity of miRNA species was found in ovary samples and the lowest in fruit samples.

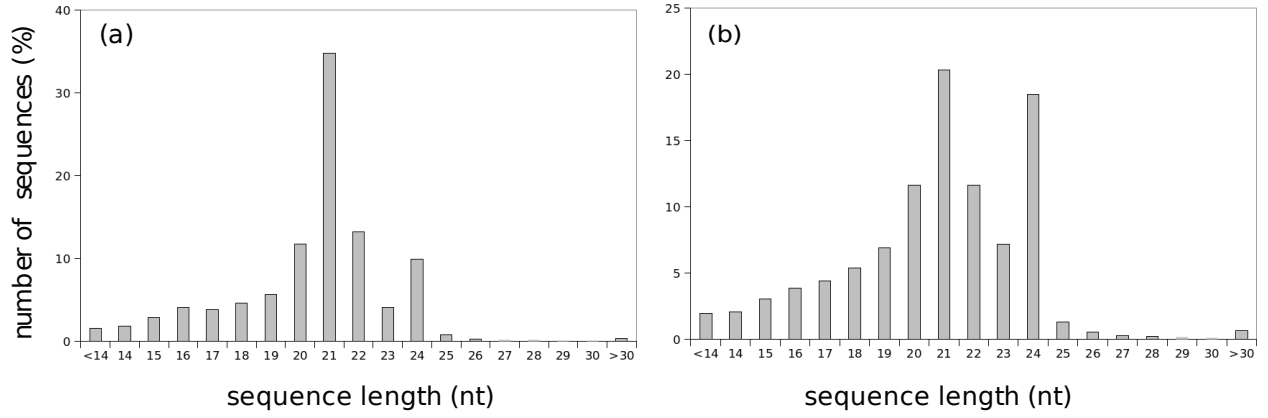

Figure 1 Representation of sequences with different lengths in the melon sRNA data set. *(a)* All sequences. *(b)* Non-redundant sequences. The number of sequences is expressed as a percentage of the total number of sequences.

Table 2 Known plant miRNAs identified in melon

Annotation	Melon sRNA sequence (5′-3′)	Similarity	Number of miRNA sequences	miRNA* sequences	Hit in melon genome[a]
miR156|a, b, c, d, e, f	UGACAGAAGAGAGUGAGCAC	100%	469	60	YES
miR157|a, b, c	UUGACAGAAGAUAGAGAGCAC	100%	269	0	YES
miR157|d	UGACAGAAGAUAGAGAGCAC	100%	19	21	YES
miR158|a	UCCCAAAUGUAGACAAAGCA	100%	1	0	–
miR159|a	UUUGGAUUGAAGGGAGCUCUA	100%	14651	0	YES
miR159|b	UUUGGAUUGAAGGGAGCUCUU	100%	18	0	–
miR159|c	UUUGGAUUGAAGGGAGCUCCU	100%	1	0	–
miR160|a, b, c	UGCCUGGCUCCCUGUAUGCCA	100%	537	0	YES
miR161|a.1	UUGAAAGUGACUACAUCGGGG	100%	6	0	–
miR161|a.2	UCAAUGCAUUGAAAGUGACUA	100%	1	0	–
miR162|a, b	UCGAUAAACCUCUGCAUCCAG	100%	825	0	YES
miR164|a, b	UGGAGAAGCAGGGCACGUGCA	100%	172	1	YES
miR165|a, b	UCGGACCAGGCUUCAUCCCCC	100%	4	0	–
miR166|a, b, c, d, e, f, g	UCGGACCAGGCUUCAUUCCCC	100%	65	27	YES
miR167|a, b	UGAAGCUGCCAGCAUGAUCUA	100%	136	0	YES
miR167|d	UGAAGCUGCCAGCAUGAUCUGG	100%	16	1	–
miR168|a, b	UCGCUUGGUGCAGGUCGGGAA	100%	967	0	YES
miR169|a	CAGCCAAGGAUGACUUGCCGA	100%	3	1	–
miR169|b, c	CAGCCAAGGAUGACUUGCCGG	100%	76	1	YES
miR169|h, i, j, k, l, m, n	UAGCCAAGGAUGACUUGCCUG	100%	83	1	YES
miR170|a	UGAUUGAGCCGUGUCAAUAUC	100%	3	0	–
miR171|a	UGAUUGAGCCGCGCCAAUAUC	100%	85	5	YES
miR171|b, c	UUGAGCCGUGCCAAUAUCACG	100%	64	0	YES
miR172|a	AGAAUCUUGAUGAUGCUGCAU	100%	85	58	YES
miR172|c, d	AGAAUCUUGAUGAUGCUGCAG	100%	4	0	YES
miR172|e	GGAAUCUUGAUGAUGCUGCAU	100%	3	0	YES
miR319|a, b	UUGGACUGAAGGGAGCUCCC	100%	2	3	YES
miR390|a, b	AAGCUCAGGAGGGAUAGCGCC	100%	32	6	YES
miR391|a	UUCGCAGGAGAGAUAGCGCCA	100%	1	0	–
miR393|a, b	UCCAAAGGGAUCGCAUUGAUC	100%	18	0	YES
miR394|a, b	UUGGCAUUCUGUCCACCUCC	100%	4	0	YES
miR396|a	UUCCACAGCUUUCUUGAACUG	100%	134	84	YES
miR396|b	UUCCACAGCUUUCUUGAACUU	100%	82	16	YES
miR397|a	UCAUUGAGUGCAGCGUUGAUG	100%	26	0	YES
miR408|a	AUGCACUGCCUCUUCCCUGGC	100%	14	1	YES
ath-miR2111a	UAAUCUGCAUCCUGAGGUUUA	100%	1	0	YES
peu-miR2910	UAGUUGGUGGAGCGAUUUGUC	100%	8	0	YES
osa-miR167d	UGAAGCUGCCAGCAUGAUCUG	100%	3401	1	YES
tae-miR395b	UGAAGUGUUUGGGGGAACUC	100%	1	0	YES
bna-miR397a	CAUUGAGUGCAGCGUUGAUGU	95%	77	0	YES
miR156|h	UUGACAGAAGAGAGUGAGCAC	95%	91	0	YES
miR156|g	ACAGAAGAGAGUGAGCACA	90%	5	0	YES
miR169|d, e, f, g	UGAGCCAAGGAUGACUUGCCU	95%	130	0	YES
miR169|d, e, f, g	UGAGCCAAAGAUGACUUGCCU	90%	112	0	YES
miR399|a	UGCCAAAAGAGACUUGCCCUG	95%	3	0	YES
miR403|a	CUAGAUUCACGCACAAGCUCG	90%	1	0	–

[a] Sequences with hit in melon genome = 'YES'; sequences with no hit = '–'

The abundance distribution of different miRNAs in each library was estimated based on sequencing frequencies as shown in Figure 2. We used sequencing data for quantitative profiling of small RNAs, though estimation of abundance based on sequencing frequencies could be misleading due to limited sequencing depth. Many miRNAs differed in abundance according to the source library. Nevertheless, most of the redundancy reflected the accumulation of miR159a, which accounted for more than 14,000 sequences in total. Figure 2A, B compares the accumulation of miRNAs in healthy *versus* WMV-infected melon tissues from

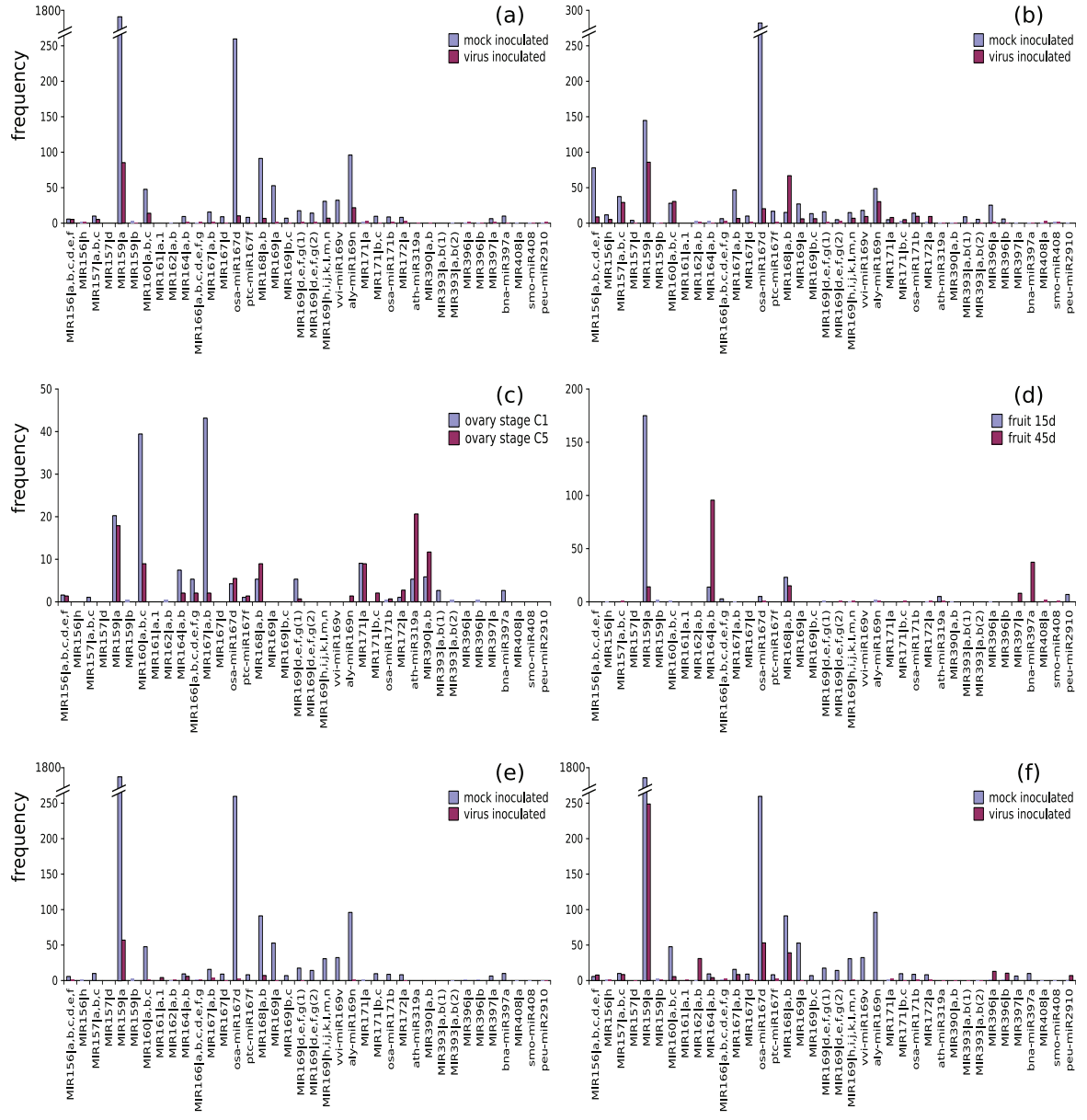

Figure 2 Relative accumulation of conserved miRNAs in melon samples used for sRNA library construction. Total reads for each miRNA in each library were normalised relative to the total number of reads from the library, and expressed per 10,000 reads. *(a)* Cotyledons from melon cv. Tendral inoculated with WMV-M116 compared to mock inoculated cotyledons of the same cultivar. *(b)* Cotyledons from the melon accession TGR-1551 inoculated with WMV-M116 compared to mock inoculated cotyledons of the same accession. *(c)* Stage C1 and C5 ovaries from melon cv. Piel de Sapo. *(d)* Fruit from melon cv. Piel de Sapo 15 days after pollination (15d) compared to fruit from the same cultivar 45 days after pollination (45d). *(e)* Cotyledons from melon cv. Tendral inoculated with MNSV-alfa5 compared to mock-inoculated cotyledons of the same cultivar. *(f)* Cotyledons from melon cv. Tendral inoculated with MNSV (chimeric virus) compared to mock inoculated cotyledons of the same cultivar.

genotypes Tendral and TGR-1551. Melon miRNA species with similarity to Arabidopsis miR156abcdef, miR160abc and miR168ab, which target mRNAs encoding squamosa promoter binding proteins, auxin response factors (ARFs) and argonaute-like proteins (AGO), respectively, showed different trends in the genotypes tested. For example, miR168ab is more abundant in healthy Tendral tissues compared to infected tissues whereas it is more abundant in WMV-infected TGR-1551 tissues than in healthy tissues. Other known miRNAs in our sequenced set were generally more abundant in healthy tissues irrespective of the melon variety tested. For example, miRNAs with similarity to Arabidopsis miR159a and miR167d, which target MYB transcription factors and ARFs, respectively, followed this trend in both genotypes albeit with differences in magnitude. Comparison of the two libraries from ovary and fruit samples (Figure 2C, D) revealed that miRNAs were particularly abundant and diverse in ovaries compared to fruits. Several miRNAs appeared to be temporally regulated during ovary development (e.g. members of the miR160, miR164, miR167, miR169, miR319 and miR390 families) whereas others were equally abundant at both ovary stages (miR156, miR167 and miR171 families). Fruits contained far fewer miRNAs than ovaries, and only miRNAs similar in sequence to Arabidopsis miR159a, miR164ab and miR397a showed significant differences in accumulation (with trends opposite to those seen in ovaries). These findings indicated that miRNAs in melon were expressed in specific tissues and in response to particular physiological conditions. In Arabidopsis, most of these miRNAs target mRNAs encoding transcription factors with roles in development, such as hormone signal transduction and organ identity. Figure 2E, F compares the accumulation of miRNAs in healthy and MNSV-infected tissues. Similar accumulation profiles were observed in both samples for most of the miRNAs identified. Exceptionally, miRNAs similar to Arabidopsis miR396a, miR396b and miR162a, which regulate transcripts encoding GRF transcription factors and DCL proteins, respectively, showed opposite accumulation patterns.

Identification of miRNA/miRNA* duplexes

DCL-mediated cleavage of miRNA precursors having the characteristic stem-loop structure gives rise to miRNA duplexes where one of the two strands is the guide miRNA (the functional molecule) while the near-perfect complement sequence is known as the passenger miRNA, or miRNA*. The miRNA* is rapidly degraded but transient species can be cloned and therefore sequenced. We identified 16 miRNA* sequences complementary to some of the 46 miRNAs in our dataset (Table 2), nine of which had the predicted sequence based on the fold-back structure of their presumptive precursors with internal mismatches and two additional terminal nucleotides forming a 3' tail (Figure 3A), whereas the other six had a different number of protruding nucleotides and were considered non-typical (Figure 3B).

The number of sequenced miRNA*s was generally much lower than the number of mature sequences but there were some remarkable exceptions. For example, for miR396a we counted 134 miRNA and 84 miRNA* sequences, as opposed to miR159a for which 14,651 miRNA sequences but no corresponding miRNA* sequences were retrieved in the sequenced collections (Table 2). The most extreme example was miR157d, for which we recovered the same numbers of miRNA and miRNA* sequences.

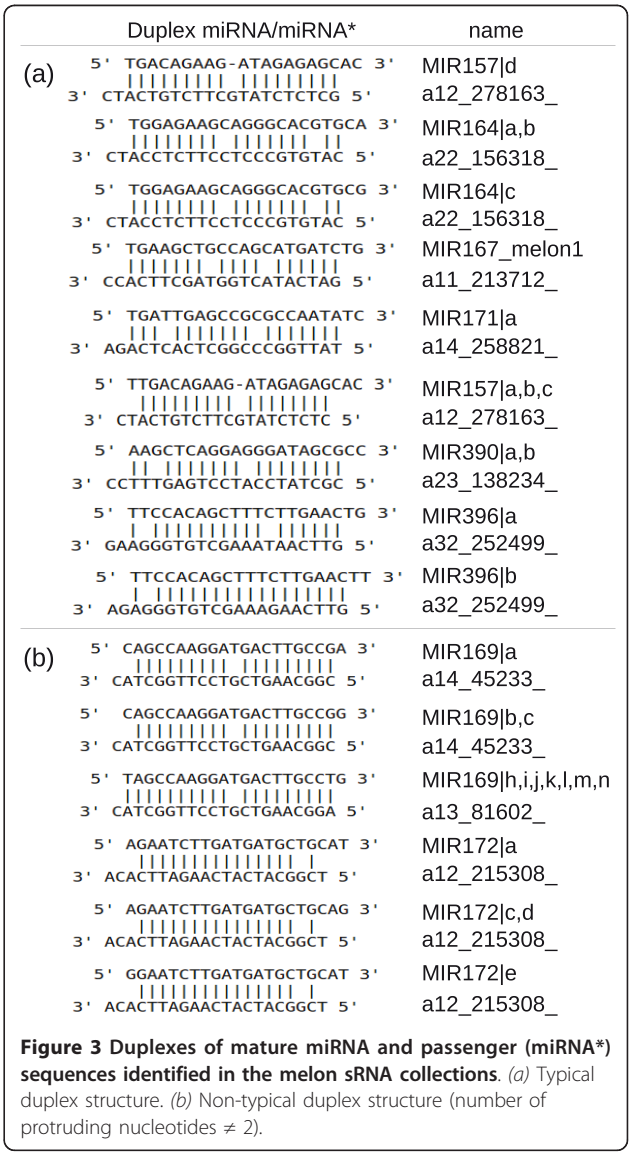

Figure 3 Duplexes of mature miRNA and passenger (miRNA*) sequences identified in the melon sRNA collections. *(a)* Typical duplex structure. *(b)* Non-typical duplex structure (number of protruding nucleotides ≠ 2).

Identification of putative melon-specific miRNAs

After identification of known miRNA sequences and other sRNA sequences (see below), 108,454 unique melon sRNAs remained unclassified, from which the most abundant (28.6%) were 24-nt species. Initial analysis confirmed that 36,783 (33.9%) of these sequences had a perfect match in the melon genome. The frequency distribution was highly skewed: 33,621 sequences had fewer than 25 hits (24,488 originated from a single locus), and only 659 sequences had more than 100 hits.

Sequences that were 21, 22 or 24 nt in length with a maximum of six hits in the genome were selected as potential novel miRNAs, and flanking genomic regions were analysed according to three consecutive criteria. First, we used miRanda software to detect sequences complementary to the potential miRNA inside the flanking regions. Second, potential miRNAs with precursors less than 70 nt in length were discarded. Finally, the MFEI index, which is used to distinguish miRNA precursors from other coding and non-coding RNAs and is based on free energy estimates and nucleotide composition [35], was calculated for each precursor and the results were sorted accordingly (the more negative the index, the better the precursor).

Predicted miRNA precursors and their genomic flanking regions that were found to be similar in sequence to previously described transposons were discarded. Other predicted miRNA precursors with intramolecular folding potential showed no similarity to known transposon sequences although their secondary structures were similar to those of known foldback transposons; these were characterised by strong negative MFEI indexes and high miRanda scores, both features consequence of high sequence complementarity in the pairing stem sequence. For some of these precursors, several uncharacterised melon sRNAs mapped on them in both the sense and antisense orientations (e.g. a11_62726 in Figure 4), up to 85 in some cases. Therefore, these were also considered unsuitable miRNA candidates. Three other potential miRNAs were shown to be the miRNA* sequences of known miRNAs that had not been picked up in our initial screen.

After manually inspecting the remaining secondary structures, 77 loci that fulfilled the structural criteria for annotation of plant miRNAs [36,37] were selected as plausible miRNA precursors; we also added to this list 7 other loci that had an asymmetric bulge involving 3 bases inside the putative miRNA duplex. From them, 43, 20 and 21 corresponded to sequenced sRNAs of 21, 22 and 24 nt in length, respectively (Table 3). Six selected sequences are shown as examples in Figure 4. By checking the pairing sequence on the stem of the predicted precursors, miRNA*s for seven candidate miRNAs were found in the sequenced set. Therefore these

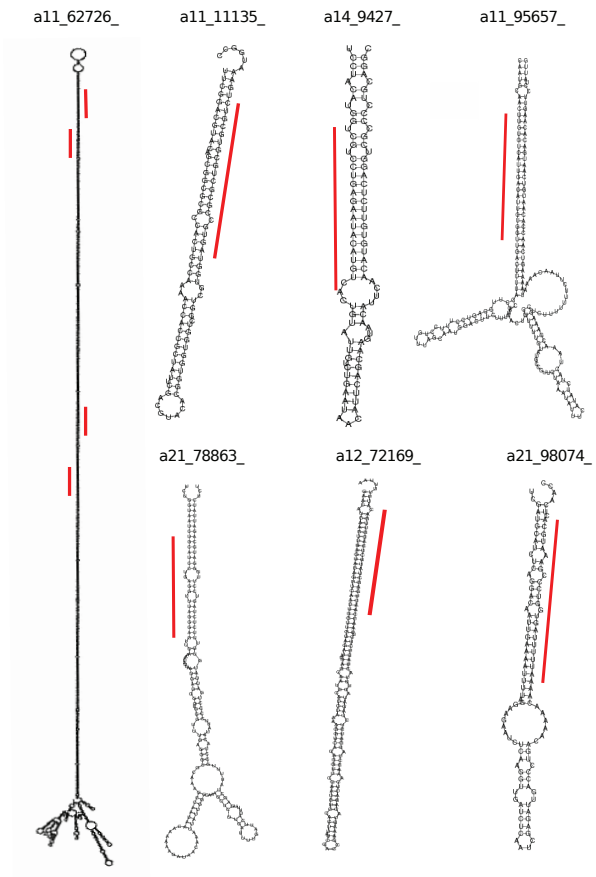

Figure 4 Secondary structures of putative novel melon-specific miRNA precursors. Some of the sRNAs identified as potentially novel and melon-specific miRNAs (listed in Table 3) were selected based on quality criteria and are shown as examples. Red lines represent regions where miRNA/miRNA* duplexes are located. Identifier a11_72726 represents an example of unsuitable miRNA candidate.

miRNAs were regarded as authentic miRNAs that conformed to the biogenesis and expression criteria for confident miRNA annotation [37]. The remaining sequences, not supported by the complementary passenger strands, were classified as candidate miRNAs. Most of the potential novel miRNA were represented by a small number of sequences, a single sequence in more than half of the cases, but six exceptional candidates were represented by more than 10 sequences (Table 3). As occurred for conserved miRNAs, sequence variants were identified for some novel miRNAs (Table 3) which mapped on the genomic melon sequence with slight variations in length and position relative to the most abundant sequence. In the absence of a reference sequence from any database, these variants were counted. Sequencing errors of differential cleavage of potential miRNA precursors possibly explain these length and positional polymorphisms.

Table 3 Potential novel melon specific miRNAs

Melon sRNA name[a]	nt	sRNA sequence	Number of sequences	Hits in genome	miRanda score[b]	Potential precursor lenght (nt)	MFEI[c]
a34_130677_	21	AUAGAUAUUGAUAUGCUUUUA	1	4	163	94	-1.0573
a24_2602_	21	UGCUACAUGGUUUAUCAGUGA	2	5	115	72	-1.2524
a24_177791_	21	UCGCAGAAGAGAUGGCGCCGA	7	1	143	91	-0.8587
a23_118111_	21	CAUUGAUAGACACUAAUAGAA	1	5	167	90	-1.475
a33_14294_	21	AUAGACUUCUAUUGGUGUCUA	1	1	154	74	-1.5105
a32_31625_	21	GUUCCCACGGUAAUGAUAAUA	2	1	127	72	-1.1045
a32_1324_	21	AGGUGUCAUCUUGCUGCGAUA	1	1	179	99	-1.2
a22_190223_	21	UGUUAUGCAUGGCGUCGGGAG	1	5	187	157	-1.2915
a14_98657_	21	AUAGCGAAGUAUAUCAGUGAU	1	1	154	127	-1.2423
a14_51701_	21	UGAGCCGUGCCAAUAUCGACG	1	1	159	97	-1.1
a14_180374_	21	UAAAUAUUUAGAAAGUCAAUC	1	4	159	79	-1.855
a21_80766_[f]	21	UAAUAUUCAUUUUCACUUCUU	1	1	116	232	-1.2494
a21_78863_	21	UGCAUCCUGAGGUUUAGGGAG	3	1	159	194	-0.9776
a21_244426_	21	AGUAACCACUAAGCUAAUGGC	1	1	187	597	-1.0096
a23_1441_[f]	21	UAUAGCAAAGUCUAUCGAUGG	5[d]	1	107	77	-1.1435
a13_84447_[e]	21	GGUCAUUCUAGCAGCUUCAAU	13[d]	1	139	201	-0.96
a23_370_[e]	21	UGGUGUGCAUGUGAUGGAAUA	13[d]	1	163	113	-0.9061
a21_134553_[f]	21	GUUAUACGAGUUGGGUUGGGU	1	1	155	114	-0.9571
a11_95657_[e]	21	UUGUGUCAUUGACAUUGUGGU	1	2	195	191	-1.1708
a14_9427_	21	UCGUCCUGAGAAUACAUGUCA	35[d]	1	159	97	-0.9409
a14_668_	21	UGAGUUAUCGGUGAAUUCAAG	5[d]	3	159	516	-0.9675
a13_252112_	21	ACUGCUGCUUGUACUAUUGAA	1	1	191	255	-1.9670
a11_33177_	21	UUUAGUUUAGCCUAUUGCUUU	1	3	187	139	-1.1035
a11_191362_[e]	21	UUCUAUUGUCUUCAUUUGUGA	1	1	191	119	-1.2718
a34_224062_	21	UGAAAUGACUUGUCAAGUGCU	1	1	151	97	-0.975
a13_228150_	21	CUUGUACUUGAUUUUGUUGCC	1	1	191	115	-1.4469
a12_32299_[e]	21	AAUUUGUUGGUCAAAUGAUUG	2	1	195	107	-1.7552
a12_272161_	21	UUGUAUGGUGGAAAGAUGGAA	1	1	162	96	-1.4167
a11_33986_	21	GCUGACUUGCUGAUUGAGUUA	2	3	179	189	-1.4852
a13_33760_	21	UGAAUUAUCUGCUUAAGUUUU	1	1	187	95	-1.2889
a11_389198_	21	ACACGCAGAAGAGACGAUUGA	1	1	191	120	-1.5575
a13_357842_	21	UGGAGCAAUAUUGAUGCAUAU	1	1	195	220	-0.9548
a11_364692_	21	UUGGGUCUAUUUAAUGGGAGC	1	1	155	107	-1.2308
a13_281334_	21	ACUUUCUGUCAAUAUAAUCAG	1	1	175	115	-1.3943
a12_71107_	21	UAUCAUAGUUGGUGGUUCAGG	3	1	143	115	-1.2167
a13_120551_	21	UCAACGAUAGACAUUGAUAGA	1	1	171	107	-1.2875
a12_123886_	21	UUAUCAUUGAUAGACUAGUAU	1	2	155	174	-1.0612
a11_227522_	21	CAAGCCCAUGACAAAGCAAGC	1	1	187	225	-1.0929
a14_133932_[f]	21	UCAACACGAUCGUCUAGCAUG	1	2	173	113	-1.2295
a11_203340_	21	UUUGAGUGUCCUACUCACCUC	1	1	191	411	-1.0833
a11_11135_	21	UAGUGCCGCGCUGCGUGCGUC	85	1	147	102	-0.98
a11_31022_	21	UUUCGCUUUUCCUCUUUCGUG	1	1	191	454	-1.1523
a12_144938_	21	UCGUGGAUAUUGCUCUUUUCU	2	1	171	504	-1.3303
a33_181157_	22	GAUAGAUACUAAUAUGCUUCUA	1	2	188	87	-1.3227
a33_37151_	22	AGAUUAAUUUAUUGGGCGUUAU	1	2	144	94	-1.3313
a12_72169_	22	UUGAGCUAUGCUCAGGUUGACA	30	1	176	174	-1.2933
a24_96796_[e]	22	UGAGCUAUGCUCGCUUUGGCAA	21[d]	1	175	169	-1.2855
a11_378153_	22	GAGUUCCUAAGUUUUGAUGAAU	1	1	144	351	-1.2221
a11_378297_	22	UUUUGGAUUCUAUCGAUGAAAG	1	1	155	123	-1.4857
a23_244052_[e]	22	GGGCAGCCCCACGUUGGGCAUG	5[d]	1	175	353	-0.9263
a11_85662_	22	AAAUAUAUCGGUGUCUAUCAAU	1	2	132	85	-1.2208

Table 3 Potential novel melon specific miRNAs *(Continued)*

a21_388555_	22	GAUAGACGCUGAUAGAUAGACA	3	1	124	76	-0.8963
a11_84237_	22	CGGCCAAAAAUGACUUGCCCGG	2	1	150	105	-0.8902
a23_124460_	22	AGGUGAGUUCUUUUUAUAGGCU	1	1	184	179	-1.6306
a23_163065_	22	AUUUGAUUAGCCAAAUUUAAAC	2	2	148	130	-1.0514
a23_71826_	22	CAAUAGUCAGAUGUAAACGAUC	1	1	180	228	-1.4282
a22_52587_	22	AAAAUUAUUGGGUGAAUUAGUU	2	1	179	162	-0.9013
a13_234225_	22	UGAAUUUUGUUAUGUUUUGUAA	1	2	168	80	-2.0417
a13_286453_	22	AGUCUAUCACCGAUAGAAGCCU	1	6	182	430	-1.4372
a21_339397_	22	CGCGAGGUUCUUUGUUUGUCUU	1	1	192	413	-1.3341
a14_283014_	22	UACCUAGUGAUGCCAUUGUCAA	1	1	184	533	-1.2829
a21_170878_	22	CUAAGGUUGCCCAGAGAUGUUC	1	1	163	271	-1.2341
a21_63125_[f]	22	GAAUAAUUAUCAAGUGUGUAGC	1	1	165	210	-1.7804
a11_146182_	24	UUAAAAUGUUGCUAUAUAAUUAAU	1	6	202	390	-1.6207
a21_169735_	24	UAUACGGGCCGUAAAUAGUUUGAU	2	6	132	369	-0.8885
a23_3672_	24	UUGCUCAUUGCUAACUGCAAAGAG	1	3	198	183	-1.7594
a14_260703_[f]	24	UUAAAAGUAUGAGACGAAAAUGAA	1	1	190	111	-1.4204
a14_218837_	24	UUAAAAAGAACUACACGAACGUGC	1	1	194	342	-1.1314
a14_148530_[f]	24	AAAUAGCGUCGGGGAAAGGUGUCU	3	1	152	73	-1.0167
a21_127379_	24	UGGGACAAAAGAAAACUGUGGGUC	2	1	162	586	-1.32
a11_2899_	24	AUGAUGCUUUGGUGCUAAGGAGGU	1	1	198	470	-1.6094
a11_248538_	24	AUUUUUGGCAUUUUACACGGUGAG	2	2	192	218	-1.5206
a13_59670_	24	AGUGGAGUGGGCUAUUUUAGUCCA	6	1	166	570	-1.1249
a32_72333_	24	AACUAUUUUAUUGGAACAUGUUGA	3	1	170	96	-1.0391
a33_22103_	24	AGUAUGAUCUCGGGCUAAGGUUGC	1	4	202	246	-1.4138
a24_224684_	24	AACAAAACGAAUGAUCAAAAUGGU	3	1	134	80	-0.8871
a13_225824_	24	ACCAAAUGGAUCUAUUCUUAUAAU	1	1	198	311	-1.3393
a24_82972_	24	AACGAUCGGGUUGACUACGUAAAU	3	1	190	146	-0.9354
a21_98074_	24	AAUUUUUAGUGGUCCCGAAAUGCA	3	1	166	112	-0.9033
a11_350684_	24	UGAGUGUAUCAUCGAGAUAGUGCG	1	1	190	307	-1.1642
a21_216237_	24	AAAUUUCAGGGUCUAAAUUGAUGC	2	1	138	447	-1.2096
a33_49599_	24	CAGCGUGAUUGAUGGGGCAUUUUU	3	1	118	287	-1.4678
a33_58155_	24	AUGGUCGAUCUCAACCGAGAUUGA	1	1	194	298	-1.3188
a23_103110_	24	CGUGAAGAUUGUGGAUAUUGGAGA	1	1	190	414	-1.5507

[a] Precursor secondary structures of sRNAs in bold are represented in Figure 4.

[b] miRanda score calculated for the identification of the complementary region to the putative miRNA.

[c] MFEI index calculated as described [35].

[d] Include cases where sequence variants with up to two mismatches were identified and counted

[e] Indicates miRNAs for which a miRNA* sequence was identified.

[f] Indicates miRNAs for which an asymmetric bulge of more than 2 bases was identified in the miRNA/miRNA* duplex of the precursor, not meeting exactly the structural criteria previously set forth [36,37].

Prediction of potential miRNA targets in the melon transcriptome

To identify potential targets of miRNAs, we screened melon unigenes in the publicly-available database [7]. Two independent searches were performed using miRanda [38] and TargetFinder [39], and the results were compared. Each program scores potential targets based on sequence complementarity, with high scores better in miRanda, and low scores better in TargetFinder. Both algorithms identified a common set of presumptive targets albeit with different scores, and the few discrepancies involved targets with low confidence scores.

Targets in Arabidopsis defined by miRanda generally have a score ≥170, and using this value as cutoff we found 150 melon unigenes as potential miRNA targets, the best of which are summarized in Table 4 (a complete list is provided in Additional file 2). The potential miRNA targets in Table 4 generally had similar annotations to their Arabidopsis counterparts, although there are some exceptions. For example, melon unigene cHS_39-F10-M13R_c is a predicted target of miR159a but it is annotated as positive regulator of brassinosteroid signaling rather than a MYB or TCP transcription factor, which is sensitive to miR159 regulation in

Table 4 Best quality miRNA targets identified in melon unigenes

miRNA annotation	Unigene	Score (miRanda)	Score (TargetFinder)	Unigene annotation
miR390|a, b	c15d_05-D02-M13R_c	362	2.5	non-annotated unigene
miR390|a, b	c15d_05-D02-M13R_c	362	4	non-annotated unigene
miR390|a, b	c15d_21-G08-M13R_c	362	2.5	non-annotated unigene
miR390|a, b	c15d_21-G08-M13R_c	362	4	non-annotated unigene
miR391|a	cCL286Contig1	325	–	histone H1, putative
miR164|a, b	cA_04-D07-M13R_c	319	–	non-annotated unigene
miR390|a, b	cCL384Contig1	316	–	ATSK11, SK 11ATSK11; protein kinase/protein serine/threonine kinase
miR391|a	cPSI_29-G09-M13R_c	313	–	UVR8UVR8 (UVB-RESISTANCE 8); chromatin binding/guanyl-nucleotide exchange factor
miR167|d	cCL1653Contig1	200	–	non-annotated unigene
miR167|d	cCL2516Contig1	200	–	non-annotated unigene
miR167|a, b	cCL1653Contig1	195	1	non-annotated unigene
miR167|a, b	cCL2516Contig1	195	0	non-annotated unigene
miR168|a, b	c46d_19-A03-M13R_c	195	0	non-annotated unigene
miR171|a	cHS_39-C12-M13R_c	195	0	scarecrow-like transcription factor 6 (SCL6)
miR397|a	c15d_32-E08-M13R_c	195	0	potential miR397a precursor
miR160|a, b, c	cCL5073Contig1	191	0.5	ARF17ARF17 (AUXIN RESPONSE FACTOR 17); transcription factor
miR164|a, b	cPSI_18-H09-M13R_c	190	3	ANAC100, ATNAC5ANAC100 (ARABIDOPSIS NAC DOMAIN CONTAINING PROTEIN 100)
miR393|a, b	cCL3757Contig1	190	2	AFB2AFB2 (AUXIN SIGNALING F-BOX 2); auxin binding/ubiquitin-protein ligasechr3
miR393|a, b	cCL4853Contig1	190	1	TIR1TIR1 (TRANSPORT INHIBITOR RESPONSE 1); auxin binding/protein binding/ubiquitin-protein ligase
miR408|a	cCL975Contig1	190	2.5	ARPNARPN (PLANTACYANIN); copper ion binding/electron carrierchr2
miR408|a	cHS_18-D07-M13R_c	190	2.5	ARPNARPN (PLANTACYANIN); copper ion binding/electron carrierchr2
miR157|a, b, c	c46d_26-C05-M13R_c	187	3	SPL4SPL4 (SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 4); DNA binding/transcription factor
miR157|a, b, c	cCl_30-A09-M13R_c	187	2	SPL9SPL9 (SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 9); transcription factor
miR157|a, b, c	cCL2877Contig1	187	3	SPL3SPL3 (SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 3); DNA binding/transcription factor
miR164|a, b	cCl_64-A04-M13R_c	187	2	NAC1, ANAC022NAC1; transcription factorchr1
miR170|a	cHS_39-C12-M13R_c	187	1.5	scarecrow-like transcription factor 6 (SCL6)
miR159|a	cHS_39-F10-M13R_c	183	3	brassinosteroid signaling positive regulator-related
miR161|a.2	cA_16-D06-M13R_c	183	3	pentatricopeptide (PPR) repeat-containing protein
miR169|a	cA_37-E12-M13R_c	183	4	NF-YA9NF-YA9 (NUCLEAR FACTOR Y, SUBUNIT A9); specific transcriptional repressor/transcription factor
miR156|a, b, c, d, e, f	c46d_26-C05-M13R_c	182	2	SPL4SPL4 (SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 4); DNA binding/transcription factor
miR156|a, b, c, d, e, f	cCl_30-A09-M13R_c	182	1	SPL9SPL9 (SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 9); transcription factor
miR156|a, b, c, d, e, f	cCL2877Contig1	182	2	SPL3SPL3 (SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 3); DNA binding/transcription factor

Table 4 Best quality miRNA targets identified in melon unigenes *(Continued)*

miR157|d	c46d_26-C05-M13R_c	182	3	SPL4SPL4 (SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 4); DNA binding/ transcription factor
miR157|d	cCl_30-A09-M13R_c	182	2	SPL9SPL9 (SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 9); transcription factor
miR157|d	cCL2877Contig1	182	3	SPL3SPL3 (SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 3); DNA binding/ transcription factor
miR319|a, b	c15d_24-H05-M13R_c	182	4	potential miR319|a, b precursor
miR167| d_melon	cCL1653Contig1	–	0	non-annotated unigene
miR167| d_melon	cCL2516Contig1	–	0.5	non-annotated unigene
miR172|e	c15d_13-C08-M13R_c	–	2	non-annotated unigene
miR172|e	cA_04-D07-M13R_c	–	2	non-annotated unigene
miR167|a, b	cCL2288Contig1	–	2.5	unknown protein
miR156|a, b, c, d, e, f	cCL5542Contig1	173	2.5	kelch repeat-containing F-box family protein
miR157|a, b, c	cCL2547Contig1	175	2.5	unknown protein
miR164|a, b	cCL2655Contig1	175	2.5	non-annotated unigene

Arabidopsis. Many of the melon unigenes identified as potential targets were not annotated, and some had previously been identified as potential miRNA precursors [5].

Interestingly, the highest miRanda scores (> 300) were achieved for transcripts with two separate miRNA targets on the same molecule. For example, unigene c15d_05-D02-M13R_c had two target sites for miR390ab separated by ~200 nt. When this region was used as a BLAST query against the melon sRNA dataset, a group of 257 sequences (more than 92% of them being 21 nt long) was identified with nearby clusters of related 21-nt sequences in both the sense and antisense orientations, which is reminiscent of the ta-siRNAs biogenesis mechanism [18] (Figure 5). Both sites (complementary to miR390 family members in unigene c15d_05-D02-M13R_c) had similar miRanda scores, they did not contain mismatches or G:U wobbles involving nucleotides 9-11 and were phased 21 nt one of each other. The number of sRNA copies was different in each cluster and were more abundant in sense orientation compared to antisense orientation. Two registers of phased 21-nt siRNAs were observed. One of them was phased with the miR390 complementary sites but the other one was not. A representative sequence from each cluster was selected and used to search for potential targets in melon unigenes, identifying > 100 transcripts with a miRanda score > 170. Several of these transcripts were annotated as ARFs and ubiquitin related gene products (Table 5).

The remaining unigenes with two predicted miRNA target sites listed in Table 4 were annotated as protein-coding transcripts and no sRNAs were identified with similarity to the region flanked by the two target sites (Figure 5), suggesting that they did not account for authentic ta-siRNA-producing loci. Targets were also sought in the reverse-complement sequences of melon unigenes, because a small proportion of the ESTs could be incorrectly oriented as an artifact of the cloning procedure [5]. Twenty-eight unigenes were identified as potential miRNA targets using the same criteria described above, most of which were found to be non-annotated (Table 6). In this new set of data, unigenes with two targets were used again as a BLAST query against the melon sRNA dataset but no hits were obtained, so these unigenes were no longer considered as potential ta-siRNAs.

With some exceptions, several miRNA targets with miRanda scores ≥170 (see Additional File 3) were identified for each of the potential novel melon-specific miRNAs listed in the previous section.

Characterization of other melon sRNAs

Next, we blasted our sRNA sequences against RNA and genomic databases to search for other sRNA species by sequence similarity (Figure 6). sRNAs similar to transfer RNA (tRNA), trans-acting siRNA, small nucleolar RNA (snoRNA) and transposons were the least abundant, whereas ribosomal RNAs (rRNA) were largely the most abundant non-coding sRNA species (Figure 6A). Intriguingly, exogenous virus-derived sRNAs were as abundant as other endogenous plant sRNAs, at least in the case of MNSV. Most of the sRNAs identified had complete sequence similarity with the reference RNA from each

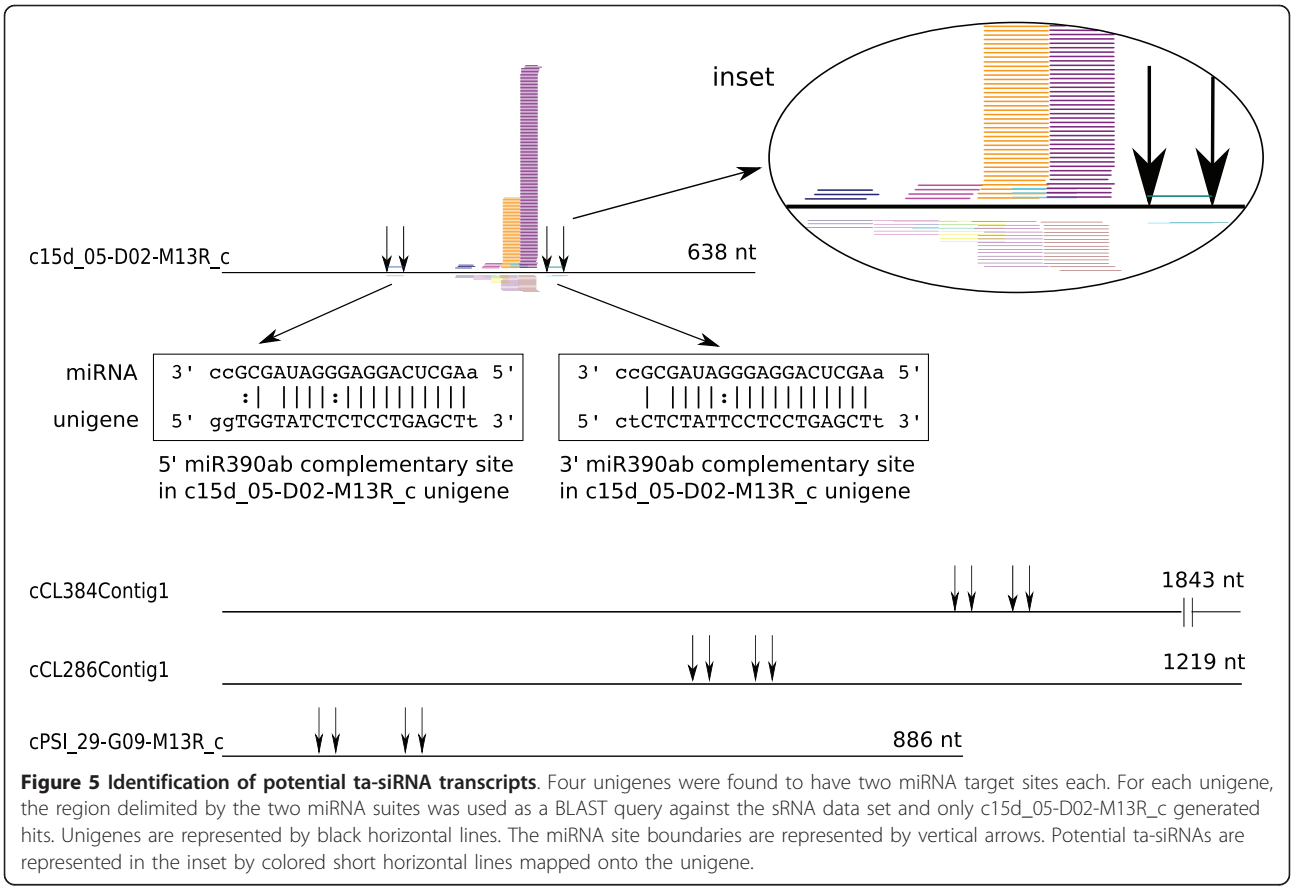

Figure 5 Identification of potential ta-siRNA transcripts. Four unigenes were found to have two miRNA target sites each. For each unigene, the region delimited by the two miRNA suites was used as a BLAST query against the sRNA data set and only c15d_05-D02-M13R_c generated hits. Unigenes are represented by black horizontal lines. The miRNA site boundaries are represented by vertical arrows. Potential ta-siRNAs are represented in the inset by colored short horizontal lines mapped onto the unigene.

Table 5 miRNA targets identified in melon transcripts for potential ta-siRNAs derived from unigene c15d_05-D02-M13R_c

Melon sRNA name	sRNA sequence	Targeted unigene	Unigene sense	Score (miRanda)	Unigene annotation
a11_156739_	AGTTTGCTTCTTGGGCTCTTC	cA_05-B09-M13R_c	Forward	175	IAA16; transcription factor
a11_156739_	AGTTTGCTTCTTGGGCTCTTC	cPS_07-G03-M13R_c	Forward	175	IAA16; transcription factor
a14_55988_	AGAGCCCAAGAAGCAAACTGG	cCL678Contig1	Forward	172	auxin efflux carrier family protein
a24_92242_	AGAGCCCAAGAAGCAAACTG	cCL678Contig1	Forward	172	auxin efflux carrier family protein
a33_151240_	CAGTTTGCTTCTTGGGCTCTT	c15d_39-H01-M13R_c	Forward	171	ARF6 (AUXIN RESPONSE FACTOR 6); transcription factor
a14_362833_	CGATGGTGATGGGATTTTTGA	cCL1479Contig1	Reverse	171	IAA9 (INDOLE-3-ACETIC ACID INDUCIBLE 9); transcription factor
a14_362833_	CGATGGTGATGGGATTTTTGA	cCL4756Contig1	Reverse	175	ATAUX2-11 (AUXIN INDUCIBLE 2-11); DNA binding/ transcription factor
a14_362833_	CGATGGTGATGGGATTTTTGA	cP5.72_c	Reverse	175	IAA7 (INDOLE-3-ACETIC ACID 7); transcription factor
a33_203464_	CATTTTTTACGATGGTGATGG	cCL3310Contig1	Forward	179	ATUBP3 (ARABIDOPSIS THALIANA UBIQUITIN-SPECIFIC PROTEASE 3)
a14_362833_	CGATGGTGATGGGATTTTTGA	cCL3310Contig1	Forward	175	ATUBP3 (ARABIDOPSIS THALIANA UBIQUITIN-SPECIFIC PROTEASE 3)
a14_20904_	TACGATGGTGATGGGATTTTT	cCL4210Contig1	Forward	174	ubiquitin-associated (UBA)/TS-N domain-containing protein
a11_156739_	AGTTTGCTTCTTGGGCTCTTC	cCL1290Contig1	Forward	171	binding/ubiquitin-protein ligase

Table 6 Best quality miRNA targets identified in reverse-complement sequences of melon unigenes

miRNA annotation	Unigene	Score (miRanda)	Score (TargetFinder)	Annotation
miR167|d	cCI_22-D03-M13R_c	327	–	metalloendopeptidase
miR390|a, b	cCL2179Contig1	317	–	ARAC1, ATGP2, ATRAC1, ROP3, ATROP3 | ARAC1; GTP binding
miR167|d	cPSI_41-B02-M13R_c	200	–	non-annotated unigene
miR157|a, b, c	cCI_04-H02-M13R_c	195	0	non-annotated unigene
miR166|a, b, c, d, e, f, g	cA_31-D02-M13R_c	195	0	non-annotated unigene
miR166|a, b, c, d, e, f, g	cCI_54-H07-M13R_c	195	0	non-annotated unigene
miR166|a, b, c, d, e, f, g	cCI_69-H04-M13R_c	195	1	non-annotated unigene
miR167|a, b	cPSI_41-B02-M13R_c	195	1	non-annotated unigene
miR168|a, b	cCI_38-C07-M13R_c	195	0	non-annotated unigene
miR170|a	cPSI_40-F10-M13R_c	191	0.5	non-annotated unigene
miR397|a	c46d_36-B03-M13R_c	191	1.5	LAC10 (laccase 10); laccase
miR157|d	cCI_04-H02-M13R_c	190	0	non-annotated unigene
miR171|b, c	cPSI_40-F10-M13R_c	190	1	non-annotated unigene
miR319|a, b	c15d_24-H05-M13R_c	190	0	non-annotated unigene
miR165|a, b	cA_31-D02-M13R_c	187	1	non-annotated unigene
miR165|a, b	cCI_54-H07-M13R_c	187	1	non-annotated unigene
miR165|a, b	cCI_69-H04-M13R_c	187	2	non-annotated unigene
miR171|a	cPSI_40-F10-M13R_c	187	2	non-annotated unigene
miR159|a	cCL1409Contig2	183	3	brassinosteroid signaling positive regulator-related
miR159|b	cCL1409Contig2	183	4	brassinosteroid signaling positive regulator-related
miR159|c	cCL1409Contig2	183	4	brassinosteroid signaling positive regulator-related
miR169|a	c15d_10-G06-M13R_c	183	4	non-annotated unigene
miR169|b, c	c15d_10-G06-M13R_c	183	4	non-annotated unigene
miR169|h, i, j, k, l, m, n	c15d_10-G06-M13R_c	183	3	non-annotated unigene
miR156|a, b, c, d, e, f	cCL1781Contig1	181	3	DNA-directed RNA polymerase II, putative (RPB10)
miR167|d_melon	cPSI_41-B02-M13R_c	–	0	non-annotated unigene
miR159|c	c15d_24-H05-M13R_c	–	2	non-annotated unigene
miR159|b	c15d_24-H05-M13R_c	–	2.5	non-annotated unigene

database (Figure 6A), even if up to two mismatches were allowed in BLAST comparisons. The only exception were sequences similar to ta-siRNAs, for which 14 melon sRNAs with similarity to Arabidopsis TAS3a|D7 (+) and TAS3a|D8(+) were identified, 2 containing 1 mismatch, and 12 containing 2 mismatches. All of them mapped very close in the melon genome and in a different region than c15d_05-D02-M13R_c unigene (the other potential source of ta-siRNAs, see above). To determine if they were authentic melon ta-siRNAs, we selected a 600 bp window sequence upstream and downstream from the genomic location determined in the melon genome for each candidate; then, a BLAST query against the melon sRNA dataset was performed, revealing that at least 126 sequences (95 of them being 21-nt in length) mapped in this region and were arranged according to a near 21-nt phase spacing (data not shown).

Many sRNA sequences also generated hits in the plastid genomes (30,239 sRNAs corresponding to 4,254 unique plastid sequences). When these sRNAs were mapped onto the melon chloroplast genome (unpublished data) (Figure 6B), two clusters of sequences resolved in regions presumably annotated as chloroplast rRNA. These regions lie within two inverted genomic repeats, and sRNAs were accordingly

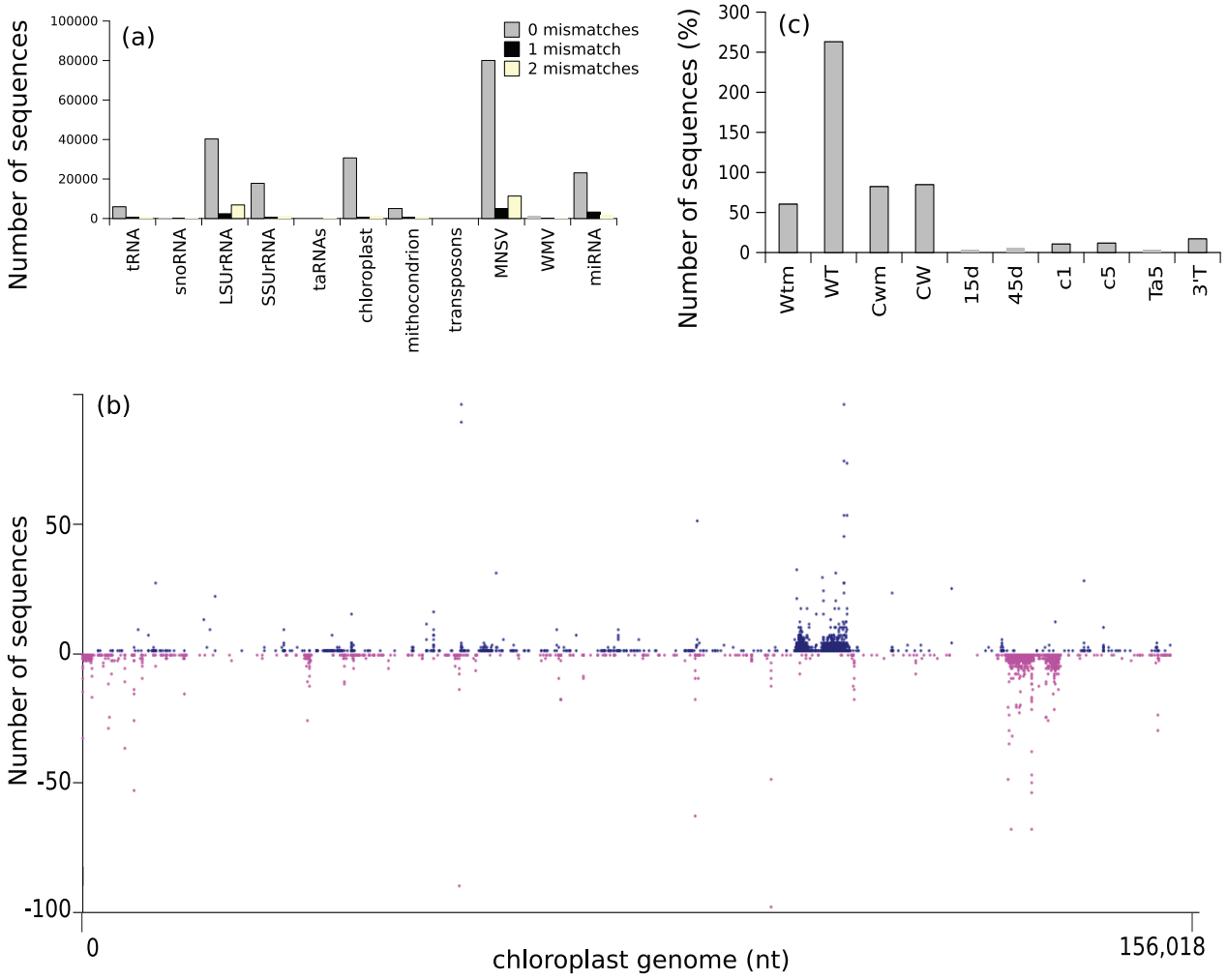

Figure 6 Types and frequencies of known melon sRNAs. *(a)* Number of sRNAs with similarity to known sequences in public databases: tRNA = transfer RNA; snoRNA = small nucleolar RNA; LSUrRNA = large subunit of ribosomal RNA; SSUrRNA = small subunit of ribosomal RNA; taRNA = *trans*-acting-si-RNAs; transposon = transposon sequences; chloroplast = melon chloroplast genome; mitochondrion = melon mitochondrial genome; MNSV = *Melon necrotic spot virus* genome; WMV = *Watermelon mosaic virus* genome; miRNA = microRNA. *(b)* Mapping of small RNAs onto the melon chloroplast genome. X-axis represents genome nucleotide positions. Y-axis represents the number of sRNAs mapped at each position. The chloroplast genome is represented by a horizontal black line (156,018 nt in length) at y = 0, and the 5′ ends of sRNAs are represented by points (blue: sense sRNAs, pink: antisense sRNAs). The frequencies of antisense sRNAs are shown as negative numbers. *(c)* Number of melon sRNAs with similarity to the melon chloroplast genome.

identified in both the sense and antisense orientations. Some of the chloroplast sRNAs had previously been cloned in other species [40]. For example, melon sRNA a33_398374 (sequence AGT TAC TAA TTC ATG ATC TGG C) was the most abundant melon plastid sRNA (18,054 counts), and a matching sequence is present in more than 900 chloroplast genomes. It is located in an intergenic region and may target a methyltrasferase transcript, although there is no direct evidence that it has silencing functions. Melon sRNA a14_392967_ (sequence GGT AGT TCG ATC GTG GAA TTT) was less abundant (166 counts), it is present in 10 different chloroplast genomes, and it may target a transcript encoding an electron carrier protein. Interestingly, different numbers of plastid sequences were obtained from each library (Figure 6C). For example, in the virus-resistant melon accession TGR-1551 there was no difference in the number of sRNAs with hits to melon chloroplast genome between healthy and virus inoculated samples, but in the virus-susceptible accession Tendral, more sRNAs were counted in inoculated samples (Figure 6C).

Unlike chloroplast sRNAs, only 7,854 sRNAs (corresponding to 2,384 unique sequences) matched the melon mitochondrial genome (unpublished data). These sRNAs were mapped on the mitochondrial genome

sequence and formed three clusters, again corresponding to the sites of rRNA genes (data not shown).

Discussion

In this report, we describe the first screen for melon sRNAs by deep sequencing. In total, 398,450 high-quality sequences were generated, representing 90% of the total raw reads. RNA species 21, 24, 20 and 22 nt in length dominated the sRNA transcriptome in melon with the 21-nt class being the most abundant in our libraries. Molecules of 24-nt processed by DCL3 are often the most abundant endogenous plant sRNAs [13], but this may vary according to species. For example, 24-nt sRNAs are more abundant in Arabidopsis, rice and tomato [41,42,9], whereas 21-nt sRNAs are more abundant in grapevine, wheat and conifers [12,43,44]. It is also possible that the composition of the sRNA population of a given plant species varies according to tissue and physiological conditions, as seems to be the case of melon (see Additional file I). Perhaps the higher proportion of 24-nt sRNAs found in melon ovaries compared to the other tissues reflects the predominance of developmental processes based on epigenetic events in the ovary.

Recent studies have shown that *cis*-acting siRNAs arising from heterochromatin, transposons and other repeat elements account for the greatest proportion of endogenous sRNA populations in plants [13,45-48]. In melon, only ~7,000 sRNAs matched known transposon sequences, in contrast to ~60,000 sRNA sequences matching ribosomal RNA, which may simply reflect the paucity of melon transposon sequence information in databases, as only 1.5% of the melon genome has been annotated for transposable elements [49]. Transposon sequences in different species show more divergence than rRNA sequences, so the representation of transposon-related sRNAs could increase when a more accurate and complete annotation of the melon genome becomes available. We also identified two sets of ta-siRNAs in our data, which mapped to different loci in the melon genome thus revealing the presence of at least two potential *TAS* genes. One locus was not represented in the melon unigene database, most likely because of its incomplete coverage. The sequence of the other locus was similar to that of a non-annotated melon transcript, and contained two registers of sRNAs in a 21-nt phase bounded by two target sites to miR390ab, reminiscent to *TAS3* genes. Non-coding transcripts containing two miR390 complementary sites that give rise to phased siRNAs have been described in other organisms. In the moss *Physcomitrella patens*, both 3' and 5' target sites are cleaved. In Arabidopsis, the 5' miR390 complementary site contains a mismatch and two G:U wobbles involving positions 9-11 and, despite it is not cleaved, it binds the silencing complex and is required for full *AtTAS3* function *in vivo* [50]. In melon, both 3' and 5' miR390 had perfect complementarity at positions 9-11, suggesting that both could be cleaved, as opposed to Arabidopsis, to specify a phased register for ta-siRNA biogenesis. Interestingly, an additional siRNA register that is likely independent of miR390-directed cleavage of the putative melon TAS transcript was observed. This alternative register might be determined by the processing activity of TAS transcripts by one of the most abundant melon primary ta-siRNAs during generation of secondary ta-siRNAs (Figure 5), as proposed for alternatively phased *TAS3* ta-siRNAs in Arabidopsis [18,50]. Since there are additional *TAS* loci in other plant genomes, it is reasonable that other melon *TAS* loci remain to be discovered.

More than 30,000 of our sRNA sequences matched the plastid genome, suggesting intense sRNA activity in this organelle. Mitochondrion-specific sRNAs were less abundant in comparison. The abundance of plastid sRNAs varied by source, with fewer sequences obtained from the ovary and fruit libraries compared to the cotyledon libraries, perhaps reflecting a relationship between chloroplast sRNA activity and photosynthesis. Interestingly, there was no significant difference in sRNA accumulation when comparing infected and healthy TGR-1551 cotyledons (resistant to WMV) whereas more sRNA accumulated in healthy Tendral (susceptible to WMV and MNSV) cotyledons than in infected ones. Whether or not this is related with the resistance phenotype is a matter of speculation.

More than 28,000 melon sRNAs in our sequenced collections matched known miRNAs in other plants, and 46 distinct melon sRNA species could be assigned to 26 known miRNA families. Although we generated a relatively low number of sequence reads, our data nevertheless were in good harmony with previous studies of miRNA profiling based on exhaustive sequencing of sRNA populations (e.g. in grapevine, 24 million reads, 26 known miRNA families and 26 non-conserved miRNA families; in tomato, 721,874 reads, 30 known miRNA families; and in orange, 13,106,573 reads, 42 highly-conserved miRNA families) [12,11,9]. This probably reflects the generally-accepted high level of expression reported for conserved miRNAs.

In addition to known miRNAs, 84 sRNA sequences derived from genomic loci with intramolecular folding capacities and not previously described as miRNAs in other plant species were predicted as potential melon-specific miRNAs. In most cases, only one sequence was counted from each of these miRNAs, which is consistent with reports suggesting that species-specific miRNAs are usually expressed at low level and in a tissue-specific manner [41]. The candidates listed in Table 2 include a

number of special cases, i.e. miRNAs with miRanda scores ≥195 and very strong secondary structures including an internal loop, resembling type III foldback transposons [51,52,9]. Although these sequences do not match known melon transposons, they were not considered as miRNA candidates because accurate homology-based transposon annotation and prediction occasionally needs to be complemented with *ab initio* approaches based on structural features [53,49]. However, even not considering this particular group, our data indicate that most of the precursors we identified are candidates to encode melon-specific miRNAs.

The accumulation of miRNAs was estimated by census sequencing and this showed that there is more miRNA diversity and that miRNAs are more abundant in ovaries than fruits. Although miRNAs are involved in many processes, 60-70% of known plant miRNAs control the expression of transcription factors that regulate critical developmental processes, such as proper specification of floral organ identity or leaf polarity, and overexpression or knockout of *MIRNA* genes led to severe developmental defects [48,54,13]. It is likely that the greater abundance of miRNAs in the early ovary stages compared to fruit reflects the more significant developmental activity in ovaries, and confirms that meristems and other developmentally active tissues are good resources for miRNA screening.

The comparison of healthy and virus-infected melon tissues showed that generally miRNAs were less abundant in infected tissues. Viruses interfere with and exploit endogenous RNA-silencing pathways using diverse strategies [55,19]. For example, the potyvirus silencing suppressor HC-Pro has been shown to suppress the miRNA pathway by inhibiting miRNA assembly into AGO1-containing silencing complexes and unwinding of miRNA/miRNA* duplexes, causing accumulation of stable duplexes [56]. Several studies have shown that virus infection can regulate the accumulation of mRNAs targeted by miRNAs without affecting the abundance of the miRNAs themselves, or even by promoting a slight accumulation [57-59]. In contrast, we found that miRNA accumulation was generally depressed in infected plants compared to controls subjected to mock inoculations. A notable exception was miR168ab, which was upregulated in the resistant genotype but downregulated in the susceptible one. This miRNA has previously been shown to be involved in controlling the expression of ARGONAUTE1 (AGO1), the catalytic subunit of the RNA-induced silencing complex responsible for slicing of target mRNAs [60]. Recent work has described the enhanced expression of miR168 and *AGO1* mRNA in virus-infected plants specifically and independently of other miRNAs [61-63]. The contrasting miRNA profiles observed in the TGR-1551 and Tendral varieties suggests that silencing may underly the resistance of TGR-1551 to WMV, although this is a hypothesis that will require further research.

We have identified more than 150 melon unigenes as potential targets for the known and novel miRNA sequences discovered in this investigation. Many animal transcripts are targets for more than one miRNA but this phenomenon is uncommon in plants [64]. Accordingly, most of melon unigenes identified as potential targets featured only a single miRNA site. miRNAs that are conserved across species tend to have conserved targets too, and our data confirm this is the case in melon. However, several unigenes predicted with high confidence as targets for conserved miRNAs had different annotations to the corresponding target genes in Arabidopsis, although these may represent false positives that would fail additional validation. Furthermore, the non-conserved targets of conserved miRNAs can be cleaved at a lower frequency than conserved targets [12]. For these two reasons, the selection of targets for individual validation experiments can be challenging.

An interesting alternative for miRNA target discovery in a genome-scale is the analysis of the small RNA degradome [65], as this avoids the *a priori* selection of potential targets. High-throughput gene expression profiling techniques such as microarray hybridization can also help to predict miRNA targets because some times a negative correlation between the abundance of miRNAs and their target mRNAs can be identified [66,67]. We have used microarrays to monitor gene expression profiles in healthy TGR-1551 and Tendral plants and plants infected with WMV. When compared with our miRNA data, we were able to identify two unigenes encoding AGO proteins that were differentially expressed and showed contrasting expression profiles in susceptible and resistant genotypes (Gonzalez-Ibeas and Aranda, unpublished data). The same profile was observed for miR168 accumulation, suggesting that miR168 may be involved in virus resistance and providing the basis for future experiments.

Conclusion

We have analysed and catalogued a collection of melon endogenous sRNA obtained through massive cDNA sequencing and have identified known miRNAs and tasiRNAs (conserved in other species) as well as potential melon-specific miRNAs with no database matches. We have also identified potential targets for these miRNAs in the melon transcriptome. Census sequencing (i.e. counting the number of sequence reads for each sRNA) was used to profile their expression in different tissues, and in healthy *vs*. virus-infected cotyledons. By comparing the predicted targets and the differential expression profiles we were able to provide insights into the role of

miRNAs in the regulation of fruit development and plant-virus interactions.

Methods

Plant material

Small RNA libraries were prepared using material from three melon accessions: 1) the Tendral cultivar (Semillas Fitó, Barcelona, Spain), which is susceptible to MNSV and WMV, 2) the breeding line T-111 of the cultivar Piel de Sapo (Semillas Fitó, Barcelona, Spain), and 3) the genotype TGR-1551 (germplasm collection of "Estación Experimental La Mayora" (EELM-CSIC), Málaga, Spain), which is resistant to WMV.

Melon plants were grown under greenhouse conditions (~25/20°C, 16-h photoperiod, ~70% relative humidity) in 0.5-L pots with substrate (Tendral and TGR-1551) or in soil bags with the capacity for four plants (Piel de Sapo). Fruits of 15 and 45 days after pollination (DAP) were collected and mesocarp tissues were recovered and used for RNA extractions. Virus infected samples were obtained from completely expanded cotyledons rubbed with carborundum (ø = 0.037 mm) and the corresponding viral inoculum. MNSV-infected melon cotyledons exhibiting lesions and marrow leafs systemically infected with WMV were ground in cold inoculation buffer (0.2 M phosphate buffer pH = 8.0, 0.1% (v/v) *beta*-mercaptoethanol, 0.03 g/ml activated charcoal) for inoculum preparation. Mock-inoculated control cotyledons were rubbed with inoculation buffer and carborundum alone.

Cotyledons were harvested at 1, 3, 5 and 7 days post-inoculation (dpi) and pooled for RNA extraction. Fruit samples were prepared as previously described [8]. Ovaries were collected at stages C1 and C5 (Mascarell-Creus et al., unpublished). The C1 stage corresponds to flower emergence from the inflorescence bud, when the outermost perianth organs commence development and no floral whorls are visible. The C5 stage corresponds to anthesis, when the flower is ready to be fertilized and all floral organs are fully formed, including the yellow petals that attract pollinators. Under normal growth conditions, C1 to C5 development takes approximately 5 days.

Small RNA library construction

Total RNA was extracted using Trizol-Reagent (Sigma Chemical Co., St. Louis, MO, USA) and 300 μg were used to construct sRNAs libraries as described [57,32]. The 3' adaptor was replaced with a pre-activated 5'-adenylated oligonucleotide (5'-rAppCT GTA GGC ACC ATC AAT 3ddC-3') (Integrated DNA Technologies, Coralville, Iowa, USA) to avoid sRNA circularisation.

Ten chimeric RNA/DNA oligonucleotide 5' adaptor variants were generated by modifying the four-nucleotide identifier (barcode): 1-1, ATC GTA GGC ACC UGA UA; 1-2, ATC GTA GGC CAC UGA UA; 1-3, ATC GTA GGC UGC UGA UA; 1-4, ATC GTA GGC GUC UGA UA; 2-1, ATC GTA GCG ACC UGA UA; 2-2, ATC GTA GCG CAC UGA UA; 2-3, ATC GTA GCG UGC UGA UA; 2-4, ATC GTA GCG GUC UGA UA; 3-1, ATC GTA GAC GCC UGA UA; 3-2, ATC GTA GAC CGC UGA UA. After each ligation step, sRNA was purified by 17% denaturing polyacrylamide gel electrophoresis. The purified, ligated sRNA was reverse transcribed with SuperScript® III reverse transcriptase (Invitrogen BV/Novex, Groningen, Netherlands) and the cDNA was amplified with AmpliTaq Gold® DNA Polymerase (Applied Biosystems, Foster City, CA, USA) using 3' PCR FusionB and 5' PCR FusionA primers [57]. The PCR primers contained the "A" and "B" tag sequences compatible with 454 technology [31].

DNA amplicons were gel-purified using 4% Metaphor Agarose and isolated using the QIAEX II Gel Extraction Kit (Qiagen, Hilden, Germany). The quantity and quality of the DNA amplicons were determined using a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA) and an Experion Automated Electrophoresis System (Bio-Rad, Hercules, California, USA). The same quantity of DNA from each library was pooled and sequenced using the 454 Life Science Technology platform (Lifesequencing S.L., Paterna, Valencia, Spain). Sequence data in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE28653 http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE28653.

Bioinformatics

The sRNA sequences were parsed from FASTA-formatted files containing 447,180 reads from two independent 454 sequencing runs and assigned to specific libraries by identifying the sRNA/adaptor boundaries and barcode analysis. Sequences were analysed with standard Python scripts [68] and the BioPython library [69]. Only sequences with the 3' and 5' adaptors in the correct position were considered. Known sRNAs were identified by searching public databases using BLAST version 2.2.19 [70] and allowing up to two mismatches. The following databases and sequences were searched: Transfer RNA Database (version 2009) [71], Plant Small Nucleolar RNA Database (v1.2) [72], SILVA (ribosomal RNA database, v100) [73], The Arabidopsis Small RNA Project (ASRP) Database [33], Rfam Database 10.0 [74], miRBase (release 16) [34], The Plant Repeat Database [75], *Cucumis melo* chloroplast genome (unpublished data), *Cucumis melo* mitochondrial genome (unpublished data), MNSV genome (GenBank accession

AY122286.1), WMV genome (GenBank accession AY437609.1). In the case of miRNAs, melon sequences were named with the reference miRNA from each database in order to distinguish miRNA species of each family. miRNA targets were identified using miRanda v3.0 [38] and TargetFinder Perl script 1.5 [39]. Putative novel melon-specific miRNA genes were identified by using the candidate miRNA as a BLAST query against the melon genome (unpublished data). For each hit, 600 bp of sequence upstream and downstream of the alignment was used to search for a near-perfect reverse complement (miRNA*) sequence with the miRanda algorithm. Regions lacking a corresponding miRNA* sequence were discarded. Minimum genomic regions (> 70 nt) containing miRNA and miRNA* sequences were selected as potential precursors. Those corresponding to protein-coding genes were identified by BLAST searches against the Arabidopsis protein database (TAIR) and were discarded, whereas non-coding potential precursors were manually inspected and used to predict the RNA secondary structure with Mfold [76] and for calculation of the MFEI index [35]. Precursors that met structural miRNA criteria were selected for further evaluation [36,37].

Additional material

Additional file 1: Length distribution of the small RNA data set for each library. Length distribution of melon sRNAs for each library (listed in Table 1). Sequence numbers are shown as a percentage of the total number of sequences obtained from every library. Data are given for total (with redundancy) and unique (no redundancy) sequences.

Additional file 2: Known miRNA targets identified in melon unigenes. Complete set of all known miRNA targets identified in melon unigenes.

Additional file 3: Novel melon-specific miRNA targets identified in melon unigenes. Complete set of all novel melon-specific miRNA targets identified in melon unigenes.

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Authors' contributions
DGI prepared RNA from infected and mock-inoculated samples, constructed all the sRNA libraries, carried out the trimming and analysis of the sRNA sequences and wrote the manuscript. JB provided bioinformatics analysis support. LD and CL provided guidance for the preparation of the sRNA libraries and additional technical support. ACD, AMC, MS and JGM prepared RNA from fruit and ovary samples. MAA supervised DGI including writing of the manuscript, and conceived this study together with DGI, JGM and CL. All authors read and approved the final manuscript.

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Publicación III.

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Aportación del doctorando: Cultivo de las plantas de melón y preparación de las muestras de RNA. Síntesis de cDNA de doble cadena por los dos procedimientos descritos en la publicación. Análisis bioinformático de los resultados de microarray bajo tutela de Joaquín Cañizares y Jose Blanca Postigo (COMAV, Valencia). Diseño experimental y escritura del mansucrito bajo tutela de los directores de Tesis.

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BRIEF COMMUNICATION

A Cost-effective Double-Stranded cDNA Synthesis for Plant Microarrays

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Abstract DNA microarrays are two-dimensional arrangements of specific probes deposited on a substrate that have been widely used in gene expression analysis by measuring mRNA accumulation. The use of this type of microarrays involves the synthesis of cDNA, which has to be double stranded (ds) if the microarray probes are of the positive strand. We have used a melon custom-synthesized noncommercial NimbleGen microarray to evaluate a modification of the SMART™ (switching mechanism at the 5′ end of the RNA transcript) procedure of ds cDNA synthesis, which differs substantially in its economical cost relative to a widely recommended method based on the nick translation approach. The results suggested that both methods produce cDNA representative of the transcriptome to a similar extent, indicating that the alternative technique provides a cheaper method of ds cDNA synthesis for plant microarray gene expression assays when the RNA starting material is not limiting.

Keywords Microarray · Double-stranded cDNA · Gene expression

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Introduction

Microarrays are two-dimensional arrangements of specific biological probes (e.g., DNA, protein, cells, or tissues) deposited on a glass slide or other substrate and are used to perform specific binding (hybridization in the case of nucleic acids) assays. Since the first DNA microarray was used (Schena et al. 1995), the technology has evolved quickly in terms of format, substrates, type of probe molecules, and the techniques for depositing them on the substrate (Barbulovic-Nad et al. 2006). During the last 10 years, DNA microarrays have become one of the most popular technologies employed for gene expression assays by measuring mRNA accumulation. To date, more than 500,000 assays have been deposited in the Gene Expression Omnibus database (GEO; Barrett et al. 2011), tens of tools for data mining have been developed (Dudoit et al. 2003; Page and Coulibaly 2008), and microarray platforms are still frequently used to perform high throughput transcriptome profiling assays in plants (Soria-Guerra et al. 2011; Stolf-Moreira et al. 2010; Yang et al. 2010) and other organisms.

Obtaining double-stranded (ds) cDNA is a requirement for the generation of labeled targets when the microarray probes are of the positive strand. Following the manufacturer's recommendations, ds cDNA can be obtained using the double-stranded cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA), based on a nick translation approach (Gubler and

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Hoffman 1983; Okayama and Berg 1982). However, cDNA synthesis by this method is expensive and considerably increases the overall cost of microarray assays. Here, we have explored and validated an alternative method of preparing ds cDNA for microarray hybridizations, based on the use of *Moloney murine leukemia virus* (MMLV) reverse transcriptase (RT), which has terminal transferase and template-switching activities (Matz et al. 1999; Zhu et al. 2001). MMLV-RT has been extensively used for the generation of full-length or near full-length cDNAs with the SMART™ (switching mechanism at the 5′ end of the RNA transcript) (Zhu et al. 2001) technology. In SMART, the terminal transferase activity of MMLV-RT primarily adds cytosine triphosphates (CTPs) to the 3′ end of the first strand cDNA (equivalent to the 5′ end of the mRNA). These CTPs then serve as the primary annealing site for a second oligonucleotide (SMART IV) with three guanine nucleotides that base pair to the C-rich region added by the RT, allowing the MMLV-RT to switch templates and continue replicating to the end of this oligonucleotide template. Critically for what is presented in this paper, after the first strand cDNA synthesis, there is one additional PCR amplification step to obtain ds cDNA.

Nucleic acid samples amplified by PCR can be subjected to PCR-inherent bias, such as preferential amplification of certain templates (PCR selection) and template reannealing with increasing PCR cycle numbers, leading to potential misrepresentations of the original transcript levels in an RNA sample. Several works have addressed this problem for technologies other than cDNA synthesis for microarray analysis (Lueders and Friedrich 2003; Mathieu-Daudé et al. 1996; Polz and Cavanaugh 1998; Suzuki and Giovannoni 1996). In order to overcome PCR limitations when mRNA amplification is needed, alternative methods have been developed, such as the T7 transcriptase-based method (Van Gelder et al. 1990) or the isothermal mRNA amplification procedure (Dafforn et al. 2004). One of the best cDNA amplification approaches uses the template-switching mechanism of the SMART procedure coupled to the T7-based amplification method (Wang et al. 2000), suggesting that the PCR step in the SMART protocol may be indeed a major drawback of the technique. In any case, amplifying the starting material by whatever method can give raise to reproducible microarray data, but may induce slight distortions relative to the initial transcript levels. Thus, several studies have pointed out caution is required when interpreting results obtained from amplification procedures (Nygaard et al. 2003; Puskás et al. 2002). If the starting material is clearly limiting, such as for samples obtained from laser capture microdissections, RNA amplification protocols might be strictly necessary; however, if the starting material is not limiting (five or more micrograms available), the need for amplification is not necessary.

Therefore, the SMART method could be modified by omitting the PCR amplification in many cases, but this step is also used, in theory, for the production of ds cDNA; a key aspect if the microarray has positive strand probes. In the present work, we show that omission of the PCR step from the SMART protocol does not compromise the generation of ds cDNA, so the method can be effectively optimized to reduce costs when the starting material is not limiting. Several MMLV-RTs have been described to have terminal transferase activity, including PowerScript (Clontech, Mountain View, CA, USA) and PrimeScript (Takara Bio Inc., Otsu, Shiga, Japan). We selected the latter for the assay described here, and microarray data generated by the PrimeScript enzyme and the Invitrogen kit were compared. For the comparison, we used a recently described melon microarray (Mascarell-Creus et al. 2009) that has a basic four-plex design and 75 K positive strand probes synthesized by photolithography. Each of the 17,444 unique tentative melon consensus sequences (unigenes) (Gonzalez-Ibeas et al. 2007) has four 60mer probes designed according to quality rules, such as non-repetitiveness (uniqueness), frequency in the transcriptome, and melting temperature. This platform has been checked and used to analyze fruit quality traits, ovary development, and pathogen resistance (Mascarell-Creus et al. 2009). Our results suggested that both methods of cDNA synthesis are comparable, leading to a similar representation of the melon transcriptome and providing a less expensive alternative that requires fewer steps and less time.

Materials and Methods

Plant Material and RNA Extraction

Melon plant growth and *Watermelon mosaic virus* (WMV) inoculations were performed as described (Gonzalez-Ibeas et al. 2011). Total RNA was extracted from photosynthetic-expanded cotyledons with the Tri-Reagent (Sigma Chemical Co., St. Louis, MO, USA), following the manufacturer's instructions. Four RNA extractions corresponding to four different cotyledons were pooled to obtain each RNA sample. To eliminate traces of genomic DNA, total RNA was incubated with DNase I (New England Biolabs, London, UK) for 10 min at 37°C. The reaction volume was then adjusted to 100 µl and the aqueous phase was extracted with phenol/chloroform/isoamyl alcohol (125:24:1). Total RNA was precipitated with 10 % (*v*/*v*) 3 M NaCl and 2.5 volumes of absolute ethanol and was centrifuged (12,000×*g*, 20 min, 4°C). Quantity and quality of RNA were estimated using an ND-1000 spectrophotometer (Nano Drop Technologies, Wilmington, DE, USA) and an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), respectively.

cDNA Synthesis and Microarray Hybridization

For synthesis of cDNA following the nick translation approach (method A), 12 μg from each RNA sample was sent for processing to the microarray hybridization service of NimbleGen (Roche NimbleGen Iceland Llc., Reykjavik, Iceland). Briefly, the process consisted of cDNA synthesis, Cy3 cDNA labeling, hybridization, scanning, and image reading. Following NimbleGen's recommendations, cDNA was synthesized using the double-stranded cDNA synthesis kit from Invitrogen (Invitrogen, Carlsbad, CA, USA). Raw and processed microarray data are freely available from GEO database under the series record GSE30693 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE30693, samples GSM761270 to GSM761305). Using this set of microarray hybridizations as a reference, two RNA samples (GEO accession numbers GSM761290 and GSM761291, samples A1 and A2 from the results section, respectively) were used for both ds cDNA synthesis methods. The SMART procedure with PrimeScript (Takara Bio Inc., Otsu, Shiga, Japan) reverse transcriptase (method B) had some minor modifications. Twelve micrograms of total RNA from each RNA sample was mixed with 2 μl of oligo(dT)-16mer (50 μM), 2 μl of SMART IV oligonucleotide 5′ AAG CAGTGGTATCAACGCAGAGTGGG 3′ (50 μM), and sterile milliQ water up to 22 μl total volume in a microcentrifuge tube (200 μl). The mixture was incubated at 70°C for 3 min and cooled on ice for 2 min. To the RNA/primer mixture, 8 μl of 5× buffer (250 mM Tris–HCl, pH 8.3, 375 mM KCl, 30 mM MgCl2, not supplied with the enzyme), 4 μl of dNTPs (10 mM each), 4 μl of DTT (100 mM), and 2 μl (400 units) of PrimeScript (Takara Bio Inc., Otsu, Shiga, Japan) reverse transcriptase were added. The mixture was incubated at 42°C for 60 min and at 70°C for 15 min. One microliter of RNAse A (20 mg/ml) (Invitrogen, Carlsbad, CA, USA) was added and incubation continued at 37°C for 10 min. From this point, NimbleGen's protocol was followed. In brief, purification of the ds cDNA consisted of the addition of sterile milliQ water up to 160 μl total volume and of 160 μl of chloroform/isoamyl/alcohol (125:24:1, *v*/*v*/*v*) for a phenolization step. The aqueous phase was retrieved after centrifugation of the mixture in a phase lock tube (Eppendorf AG, Hamburg, Germany). To the aqueous phase, 16 μl of ammonium acetate (7.5 M), 7 μl of glycogen (5 mg/ml), and 330 μl of absolute ethanol were added. The mixture was centrifuged (12,000×*g*, 20 min, 4°C). Supernatant was discarded and 500 μl of 80 % ethanol was added before a second centrifugation step (12,000×*g*, 5 min, 4°C). The pellet was rehydrated in 20 μl of sterile milliQ water. The quantity and quality of cDNA were estimated using an ND-1000 spectrophotometer (Nano Drop Technologies, Wilmington, DE, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) with a 7500 DNA chip, respectively. Double-stranded cDNA was sent for further processing to the microarray service of the Institute of Research in Biomedicine (Barcelona, Spain). The processing consisted of Cy3 cDNA labeling, hybridization, scanning, and image reading. Raw and processed microarray data are freely available from the GEO database under the same series record (GSE30693, http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE30693) and GEO accession numbers GSM761306 and GSM761307 corresponding to samples B1 and B2, respectively.

Duplex-Specific Nuclease Treatment and DNase I Digestion

For duplex-specific nuclease (DSN) (Shagin et al. 2002) treatment, 1 μg of cDNA was mixed with 5 μl of 2× master buffer (supplied with the enzyme), 2 μl of DSN enzyme (2 units) (Evrogen, Moscow, Russia), and sterile milliQ water up to 10 μl total volume. The mixture was incubated at 65°C for 10 min, and the reaction was stopped with 10 μl of 5 mM EDTA. For DNase I treatment, 1 μg of cDNA was mixed with 1 μl of 10× buffer (supplied with the enzyme), 1 μl of DNase I enzyme (2 units) (New England Biolabs, London, England), and sterile milliQ water up to 10 μl total volume. The mixture was incubated at 37°C for 10 min. For negative controls for both treatments, the enzyme was replaced by water. Treated cDNAs shown in Fig. 1a were resolved by electrophoresis on a 1 % agarose gel and stained with ethidium bromide. PCR was performed with a standard DNA polymerase (Biotools, Madrid, Spain) following the manufacturer's instructions, using 20 ng of treated cDNA as the input. The amplified PCR product (a 100-bp amplicon) corresponded to a melon *CYCLOPHILIN* transcript (unigene cCL3169Contig1, Melogen database (Gonzalez-Ibeas et al. 2007)). Primers used were: forward primer 5′ CGATGTGGAAATTGAC GGAA 3′ and reverse primer 5′ CGGTGCATAAT GCTCGGAA 3′. PCR products shown in Fig. 1b were resolved by electrophoresis on a 2 % agarose gel and stained with ethidium bromide.

Bioinformatic Analysis

Data produced by the NimbleGen service were normalized using the normalization algorithm RMA within the package Oligo (v 1.8.2) (Carvalho et al. 2007) written in R (v 2.9.1) (R Development Core Team 2010). Sample box plot diagrams were generated using this same package. Biological variability was estimated using the module principal component analysis (PCA) (Raychaudhuri et al. 2000) of the Multiexperiment viewer (v 4.4.1) (Saeed et al. 2006). Expression data analyzed by probe,

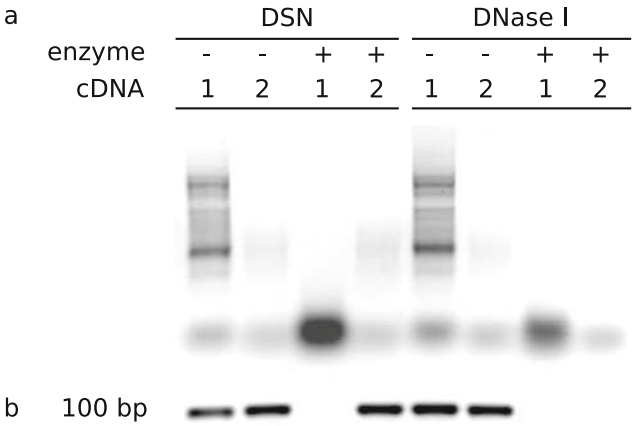

Fig. 1 Verifying that the cDNA synthesized by the SMART procedure is double stranded. **a** cDNA was synthesized by the SMART procedure in the presence of the SMART IV oligonucleotide (cDNA1, expected to be double stranded (ds)) and in the absence of the SMART IV oligonucleotide (cDNA2, as a single-stranded (ss) cDNA control). cDNAs were treated with DSN, a DNase which specifically cleaves ds DNA, and DNase I, which cleaves double- and single-stranded DNA. The ss cDNA electrophoresis pattern was difficult to stain and distinguish on an agarose gel; therefore, the presence of a PCR product from the treated cDNA was used as an additional test of cDNA integrity **b**. No PCR product was obtained from cDNA generated by the alternative method after treatment with DSN, suggesting effective digestion and, therefore, that the cDNA was double stranded

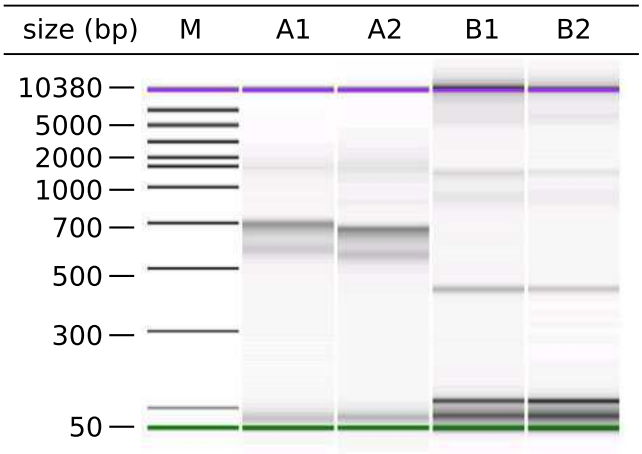

Fig. 2 Electrophoretic pattern of the cDNAs. cDNA integrity was assayed by a bioanalyzer and pseudo-gel images are shown. *First lane*: molecular weight marker. *Second lane*: sample A1. *Third lane*: sample A2. *Fourth lane*: sample B1. *Fifth lane*: sample B2. Samples *A1* and *A2*: cDNA synthesized by the nick translation approach (method A, Invitrogen's kit), samples *B1* and *B2*: cDNA synthesized by the modified SMART procedure (method B, Takara's enzyme)

probe localization, and unigene sequences were analyzed with standard Python (http://www.python.org/) (v 2.6.2) scripts and the BioPython (http://biopython.org/wiki/Main_Page) (v 1.49) library.

Results and Discussion

Recently, we have performed a melon transcription profile in response to WMV infection (Gonzalez-Ibeas et al. 2011). Cotyledons of two genotypes of melon were virus inoculated and transcriptomic responses to the infection were analyzed by comparing infected and mock-inoculated samples at 1, 3, and 7 days post-inoculation (dpi). Three biological replicates were performed for each sample. In that work, ds cDNA was obtained with the double-stranded cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA) following NimbleGen's recommendations, based on the nick translation approach (Okayama and Berg 1982; Gubler and Hoffman 1983). By using this set of microarray hybridizations as a reference, two RNA samples (replicate 3 at 1 dpi (A1) and replicate 1 at 3 dpi (A2)) were also used to perform cDNA synthesis by the alternative method (samples B1 and B2, respectively), based on the modified SMART approach (Zhu et al. 2001) presented in this work (method B).

According to the original SMART protocol, after the first strand synthesis, there is one additional step to obtain ds cDNA based on PCR amplification. However, preliminary experiments suggested that MMLV retrotranscriptase was able to generate ds cDNA on its own (results not shown); therefore, we hypothesized that this additional PCR step could be omitted. To evaluate this possibility, the obtained cDNA (method B) was checked for its double-stranded nature before microarray hybridization by treatment with a DSN (Shagin et al. 2002) that specifically cleaves double-stranded DNA, but not single-stranded DNA. The obtained cDNA was efficiently digested after treatment (Fig. 1), strongly suggesting that it was double stranded. As a control, single-stranded cDNA was obtained by a reverse transcription reaction without SMART IV oligonucleotide, and no cDNA degradation could be observed after treatment with this nuclease (Fig. 1).

Integrity of the ds cDNA obtained by both methods was checked using a bioanalyzer (Fig. 2). Regarding cDNA yield, only slight differences were observed when comparing both techniques. On average, 5 and 7 μg were obtained by using the Invitrogen's kit and the modified SMART procedure,

Table 1 Pearson's correlation coefficients of microarray data between samples obtained by alternative cDNA synthesis methods

Samples[a]	Analysis by microarray probes				Analysis by unigene (probe averaged)
	Probe 1	Probe 2	Probe 3	Probe 4	
A1–B1	0.88	0.89	0.91	0.88	0.97
A2–B2	0.9	0.91	0.91	0.91	0.97

[a] Samples A1 and A2: cDNA synthesized by the nick translation approach (Invitrogen's kit), samples B1 and B2: cDNA synthesized by the SMART procedure (Takara's enzyme)

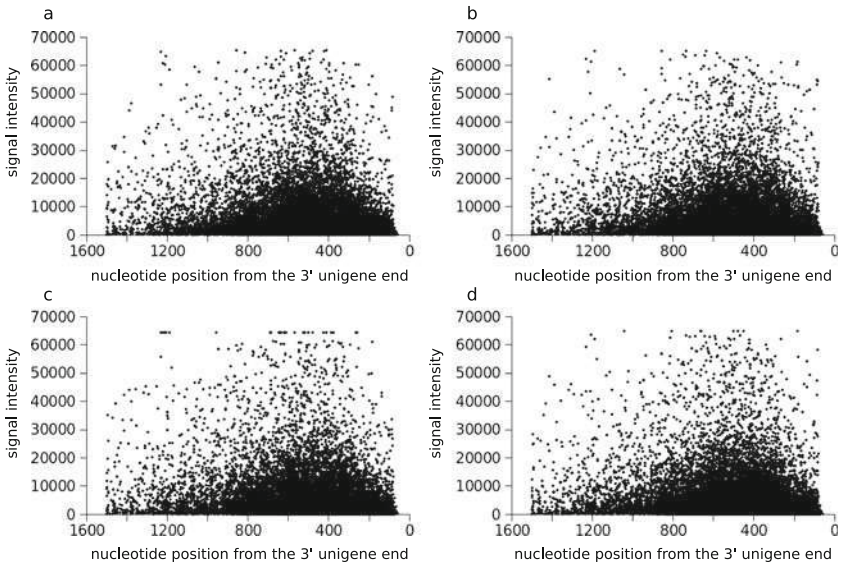

Fig. 3 Representation of the microarray signal intensities relative to their position from the 3′ mRNA end. A set of 3,965 unigenes with poly(A) tails was selected and the position of the microarray probe in the unigene sequence from the 3′ end was calculated. Normalized signal intensities for every nucleotide position are represented on the *Y*-axis. **a** Sample A1. **b** Sample B1. **c** Sample A2. **d** Sample B2. Samples A1 and A2: cDNA synthesized by the nick translation approach (Invitrogen's kit). Samples B1 and B2: cDNA synthesized by the modified SMART procedure (Takara's enzyme)

respectively, using 12 μg of total RNA as a starting material in both cases. The cDNA was sent to NimbleGen custom design service for labeling (1 μg is required), sample hybridization, microarray image scanning, and processing. The microarray images generated were manually inspected and no bulges or technical artifacts were observed. Despite full-length cDNAs can be obtained by both techniques (Gubler and Hoffman 1983; Okayama and Berg 1982; Zhu et al. 2001), we checked whether unigene sequences are represented to the same extent (5′ and 3′ ends) by both cDNA synthesis methods. Microarray signal intensities were analyzed by probe because each unigene is mapped by four probes and hypothetical misrepresentation of the 5′ mRNAs in the cDNA by one method could be reflected in different signal intensities according the position of the probe. Probes were sorted and correlation coefficients of signal intensities between both methods generated similar results (Table 1), suggesting that no probe-dependent differences occurred. However, because not all microarray probes are uniformly distributed along a unigene sequence, a second correlation analysis was performed where signal intensities were considered relative to the distance from the 3′ end of the sequence. For this task, only unigenes for which a poly(A) tail (at least 20 consecutive adenines at the end of the sequence) was present were selected and only those unigenes that were annotated in the Melogen database were used. Thus, only potential protein-coding transcripts were analyzed. A set of

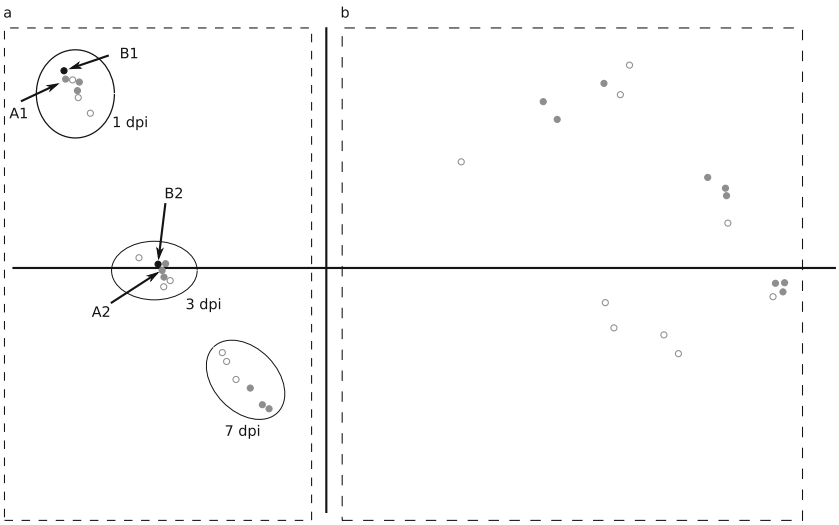

Fig. 4 Analysis of biological variability and transcriptome representation of microarray samples. PCA of healthy (*gray empty circles*) and-virus infected (*gray filled circles*) cotyledon samples at 1, 3, and 7 dpi for two melon genotypes analyzed by microarray. **a** Genotye 1. **b** Genotype 2. *Dashed lines*: samples grouped by genotype. *Black continuous lines*: samples grouped by dpi. For all these samples, double-stranded cDNA was synthesized by method A (nick translation approach, Invitrogen's kit). Two RNA samples were selected (A1 and A2) to also synthesize double-stranded cDNA by method B (modified SMART approach, Takara's enzyme, samples B1, B2, represented by *black filled circles*)

3,965 unigenes ranging from 400 to 1,536 nt, with an average size of 786 nt, was selected. When the signal intensities were mapped according to the position from the 3′ end, a minor representation of the 5′ mRNA end was observed; however, this was similar for both methods (Fig. 3).

Finally, probe signal intensities were averaged by unigene and samples were normalized in a standard microarray data processing, as previously described (Mascarell-Creus et al. 2009). After normalization, box plots revealed that most technical differences in signal intensities were eliminated (data not shown). Pearson's correlation coefficient of signal intensities was 0.97 for both cases (samples A1–B1 and A2–B2). Biological variability of normalized samples was assessed by PCA (Raychaudhuri et al. 2000). Technical replicates obtained by the alternative method of cDNA synthesis grouped very close to samples generated by the first method (Fig. 4). Indeed, despite a low dispersion being observed between samples generated by method A, and small transcriptomic changes resulting from infection being identified (Gonzalez-Ibeas et al. 2011), the new method seemed to be able to distinguish these small differences.

As with the NimbleGen, other microarray providers recommend ds cDNA synthesis kits based on the same approach. The process involves three major steps. In contrast, modification of the SMART approach, as proposed in this work, requires only 2 μl (400 units) of enzyme and the reaction takes place in a single tube in one step. Regarding the starting material, 12 μg of total RNA was used as an input for cDNA synthesis. Usually, an RNA extraction from 100 mg of melon tissue yielded 30–35 μg, and since several extractions were used in the work as a pooling strategy to reduce variability, over 90 μg of total RNA was available for experiments. This is probably the case for most plant assays and experimental systems for which starting material is not limiting and, therefore, the amplification techniques are not needed.

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Discusión general y conclusiones

Los resultados presentados en esta Tesis aportan un análisis de dos porciones del transcriptoma de melón: la que corresponde a transcritos que codifican proteínas mediante un microarray de DNA, y la que corresponde a pequeños RNAs no codificantes mediante secuenciación masiva. Ambas suponen un primer abordaje funcional al contenido genético de una especie de importancia agronómica como es el melón, que además posee características que le dan atractivo para convertirse en organismo modelo para el estudio de rasgos de interés comercial en agricultura. El valor funcional de la información generada se pone de manifiesto en que los datos de ESTs y sRNAs han servido para el entrenamiento del *software* empleado en la predicción computacional de regiones codificantes en el genoma de melón, y para la aportación de validación experimental en la identificación de genes de miRNAs, fuera del marco de trabajo de esta Tesis y dentro del proyecto de secuenciación del genoma de melón, actualmente completado (http://melonomics.upv.es/). De todas las herramientas moleculares que se han generado, quizás una de las más importantes es el microarray. El alto número de unigenes identificados tras la secuenciación de los ESTs (Gonzalez-Ibeas et al. 2007) permitió superar las espectativas del proyecto original (4.000 unigenes) y construir un microarray de *oligos* en lugar de un microaray de cDNA. Además, el alto número de unigenes ha tenido como consecuencia que las categorías funcionales de los productos génicos (términos GO) estén representadas de forma homogénea y casi en su totalidad de forma parecida a Arabidopsis (Gonzalez-Ibeas et al. 2007)), donde probablemente se conoce el transcriptoma completo o casi completo, por lo menos en lo referente a transcritos que codifican proteínas. Este hecho es de especial importancia porque, al partir de sólo 8 genotecas de cDNA y mayoritariamente procedentes de muestras de melón infectadas con patógenos y de fruto (Gonzalez-Ibeas et al. 2007)), era presumible un sesgo hacia categorías funcionales relacionadas con respuesta a patógenos y/o maduración de fruto. De esta forma, el microarray utilizado para el análisis en la Publicación I resulta más interesante para que pueda también ser utilizado para otros tipos de muestra.

En el trabajo presentado en esta Tesis, el chip se ha usado para analizar la resistencia a WMV de la varidedad de melón TGR-1551, comparando los cambios de expresión asociados a la infección viral con una variedad susceptible a dicho virus. El aspecto central de los resultados ha sido la identificación de una respuesta defensiva de la planta asociada a una resistencia recesiva. Este descubrimiento es particularmente significativo porque el paradigma de funcionamiento de las

resistencias recesivas esencialmente consiste en que los genes que confieren este tipo de resistencia codifican factores de susceptibilidad a virus, y la resistencia viene dada por la imposibilidad de complementación de las funciones virales por parte de factores de la planta, más que por la puesta en marcha de una respuesta defensiva. Por otra parte, la identificación de componentes de la maquinaria de silenciamiento génico desregulados de forma diferencial entre los dos genotipos resulta interesante por dos razones. En primer lugar, aporta un elemento adicional para ser evaluado como responsable de la resistencia, alternativo a los expuestos en el trabajo, o complementario, ya que Diaz-Pendon y colaboradores especularon sobre la existencia de elementos adicionales con un efecto epistático sobre el gen recesivo de resistencia, debido a la imposibilidad de ajustar los resultados de segregación al modelo de un único gen (Diaz-Pendon et al. 2005). En segundo lugar, los transcritos que codifican proteínas tipo Argonauta (AGO) y que se identificaron como desregulados en el microarray consecuencia de la infección viral de forma diferencial entre los dos genotipos no tuvieron bastante peso estadístico para que términos GO relacionados con este tipo de genes fueran identificados como significativos en los resultados (Publicación I). La acumulación diferencial de miR168 identificada en los datos de secuenciación masiva (Publicación II) fue el punto de partida para buscar y comparar elementos relacionados con silenciamiento génico entre las dos variedades de melón, y analizar así su posible implicación en la resistencia de TGR-1551 a WMV. A pesar del potencial que tienen las técnicas de alto rendimiento para analizar procesos biológicos, este resultado pone de manifiesto la utilidad que puede tener usar diferentes estrategias y su posterior puesta en común en el cotejo de datos durante el análisis, y viene a corroborar la utilidad de las estrategias basadas en integrómica durante el estudio de infinidad de procesos biológicos (Venkatesh and Harlow 2002).

Los resultados de secuenciación masiva en el análisis de los pequeños RNAs han mostrado que la aproximación seguida durante la construcción de las genotecas ha permitido muestrear varios tipos de pequeños RNAs, tanto endógenos de la planta como exógenos. En referencia a estos últimos, los pequeños RNAs derivados del genoma viral en plantas infectadas se han usado para el estudio de su biogénesis, fuera del marco de esta Tesis y como fruto de la colaboración con el grupo del Dr. César LLave (CIB-CSIC, Madrid). Esta aproximación resulta muy interesante porque puede usarse con otros fines, como por ejemplo la determinación de la secuencia de genomas virales para la identificación de nuevos virus y/o aislados virales (Wu et al. 2010). A pesar de la potencial implicación de la maquinaria de silenciamiento génico en la resistencia de TGR-1551 a WMV, el nivel de acumulación de sRNAs derivados del virus correlacionó con el nivel de acumulación de

RNA viral (datos no mostrados), sugiriendo que la resistencia podría estar actuando a otro nivel diferente del de la generación de viRNAs. En lo referente pequeños RNAs de la planta, en general, se puede describir en melón un contenido en sRNAs similar a otras especies de plantas, tanto en tipos como en frecuencias. Por ejemplo, mayoritariamente se ha secuenciado sRNAs derivados de regiones repetitivas, los microRNAs, ta-siRNAs y otros sRNAs se han encontrado guardando proporciones entre ellos de forma similar a otras plantas, y los miRNAs conservados con otras especies vegetales se han identificado con un nivel de expresión (en base al número de secuencias) más alto que los potencialemte específicos de melón. A pesar de los elementos comunes, siempre se encuentran elementos propios que justifican la inversión que se lleva a cabo en organismos de interés agronómico. Como ejemplos, los potenciales miRNAs específicos de melón, o el mecanismo de procesado del gen TAS descrito en la Publicación II, donde los resultados de secuenciación sugieren un procedimiento alternativo al de Arabidopsis. Un resultado menos explorado en otros trabajos ha sido la identificación de sRNAs derivados de genomas organulares. El primer detalle interesante es la asimetría en el número de secuencias obtenidas entre cloroplasto y mitocondria, sugiriendo que la actividad de sRNAs podria ser más intensa en un orgánulo que en otro. Sin embargo, es necesario matizar que similaridad de secuencia no implica necesariamente biogénesis. Aunque este apartado no se desarrolló en profundidad en la publicación, una parte de los sRNAs identificados en cloroplasto estuvieron representados también en el genoma nuclear, así que no se puede distinguir si estos sRNAs se generan desde el genoma de cloroplasto o son importados a él, tal y como sucede con muchas funciones de este orgánulo. Actualmente no hay descritos mecanismos de importación de pequeños RNAs a través de la membrana de cloroplastos, y en algunos trabajos, el material de partida para estudiar sRNAs han sido cloroplastos purificados y esto ha conducido igualmente a su identificación, sugiriendo ambas cosas que hay actividad de estas moléculas en este orgánulo. En caso de no importarse, otra pregunta interesante es, además de la función que ejercen en el orgánulo, el mecanismo de biogénesis, porque no hay descritos productos génicos del genoma organular con similaridad a, por ejemplo, proteínas tipo DCL.

Por último, las herramientas moleculares y bioinformáticas empleadas en los principales trabajos (Publicaciones I y II) se han usado para evaluar y poner a punto un método económico de síntesis de cDNA de doble cadena para hibridaciones en microarray (Publicación III). En concreto, estas herramientas son el microarray, la retrotrananscriptasa usada para la construcción de las genotecas de cDNA, la DNasa específica de dúplex usada en la normalización de dichas genotecas, y las herramientas computacionales puestas a punto en el trabajo de pequeños RNAs. A modo ilustrativo,

en el trabajo descrito en la Publicación I se llevaron a cabo 60 hibridaciones en microarray. El kit empleado para la síntesis de cDNA tenía un coste de 150 euros/reacción, de forma que se gastaron aproximadamente 9.000 euros en la síntesis. El método que se detalla en la Publicación III tiene un coste de 18 euros/reacción, de forma que la síntesis de cDNA hubiera costado 1.200 euros. Este tercer trabajo denota la utilidad de emplear las técnicas y la información metodológica de la que uno dispone en el día a día para habilitar herramientas alternativas que puedan optimizar el trabajo, en este caso desde un punto de vista económico.

Por tanto, y en conjunto, los datos presentados suponen una descripción del transcriptoma codificante y de pequeños RNAs de melón, y se ha aportado informacion para una mejor comprensión y caracterización de la resistencia de TGR-1551 a WMV.

CONCLUSIONES

1. Se ha muestreado el transcriptoma de melón correspondiente a RNAs que codifican proteínas hasta completar un total de 17.444 secuencias consenso únicas (unigenes), lo que viene a representar tres cuartas partes del transcriptoma total estimado de melón, en base a las predicciones computacionales por comparación con los genomas de otras especies de plantas.

2. El transcriptoma secuenciado, aunque incompleto, es representativo de la mayor parte de las categorías funcionales de productos génicos que describen un transcriptoma completo de plantas, sin sesgos cuantitativamente notorios en base al tipo de muestras usadas para la construcción de las genotecas.

3. En base a la información generada, se han desarrollado herramientas en genómica funcional en melón tal como una base de datos para el acceso y consulta de las secuencias, polimorfismos de secuencia para la generación de marcadores moleculares, datos sobre el uso preferencial de codones e información funcional sobre regiones codificantes, y un microarray de DNA para análisis de expresión génica de alto rendimiento.

4. Se ha muestreado el transcriptoma correspondiente a pequeños RNAs no codificantes. Se han generado secuencias de pequeños RNAs exógenos derivados del genoma de WMV y del virus de las manchas necróticas del melón (MNSV) que han servido para estudiar su biogéneis. Se han generado

secuencias de pequeños RNAs endógenos que describen la composición de esta porción del transcriptoma de melón, incluyendo miRNAs, ta-siRNAs, siRNAs y otros potencialmente relacionados con genomas organulares.

5. Se ha analizado la resistencia a WMV en una variedad resistente (TGR-1551) a este virus usando el microarray de melón previamente generado. Se ha identificado la activación de genes de defensa asociada a una resistencia recesiva, lo cual contrasta con la naturaleza de este tipo de resistencia a virus descrita en la literatura.

6. En base a los resultados del microarray y de muestreo del transcriptoma de pequeños RNAs, se ha identificado que la maquinaria de silenciamiento génico puede estar implicada en la resistencia de TGR-1551 a WMV, aportando información para una mejor caracterización de esta resistencia.

7. Se ha llevado a cabo una puesta punto metodológica en la síntesis de cDNA de doble cadena empleando las herramienta moleculares y bioinformáticas usadas previamente en este trabajo que ha permitido, en primer lugar, demostrar que la retrotranscriptase empleada es capaz de sintetizar cDNA de doble cadena por sí misma, útil para hibridaciones en microarray, y en segundo lugar, habilitar un método económico para el mismo fin cuando el RNA de partida no es limitante.

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107 (4): 1606-1611. doi:10.1073/pnas.0911353107.

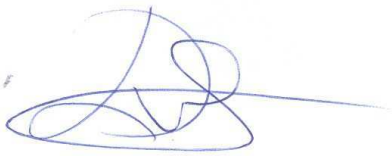
Anexo I: Cartas de conformidad de los coautores

Relación de las cartas de consentimiento firmadas por los autores de las publicaciones que componen la Tesis necesarias para cumplir los requisitos administrativos establecidos por la Universisdad de Murcia para presentar la Tesis en formato de compendio de publicaciones.

A quien pueda concernir:

Como coautor/a del trabajo "Gonzalez-Ibeas, Daniel, José Blanca, Livia Donaire, Montserrat Saladié, Albert Mascarell-Creus, Ana Cano-Delgado, Jordi Garcia-Mas, Cesar Llave, and MA Aranda. 2011. **Analysis of the melon (*Cucumis melo*) small RNAome by high-throughput pyrosequencing.** *BMC Genomics* 12: 393." doy mi consentimiento para que éste se presente como parte de los trabajos de la tesis del solicitante D. Daniel González Ibeas. Las actividades que el doctorando ha realizado en el marco de dicho trabajo no se han presentado ni se van a presentar como parte de otra tesis doctoral. Asimismo, declaramos de relevancia la contribución del doctorando en la investigación cuyos resultados se plasman en el citado trabajo.

Madrid, 1 de Marzo de 2012



Firma: Livia Donaire Segarra

A quien pueda concernir:

Como coautor/a del trabajo "Gonzalez-Ibeas, Daniel, José Blanca, Livia Donaire, Montserrat Saladié, Albert Mascarell-Creus, Ana Cano-Delgado, Jordi Garcia-Mas, Cesar Llave, and MA Aranda. 2011. **Analysis of the melon (*Cucumis melo*) small RNAome by high-throughput pyrosequencing.** *BMC Genomics* 12: 393." doy mi consentimiento para que éste se presente como parte de los trabajos de la tesis del solicitante D. Daniel González Ibeas. Las actividades que el doctorando ha realizado en el marco de dicho trabajo no se han presentado ni se van a presentar como parte de otra tesis doctoral. Asimismo, declaramos de relevancia la contribución del doctorando en la investigación cuyos resultados se plasman en el citado trabajo.

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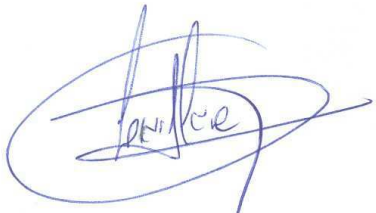

Firma: César LLave Correas

A quien pueda concernir:

Como coautor/a del trabajo "Gonzalez-Ibeas, Daniel, José Blanca, Livia Donaire, Montserrat Saladié, Albert Mascarell-Creus, Ana Cano-Delgado, Jordi Garcia-Mas, Cesar Llave, and MA Aranda. 2011. **Analysis of the melon (*Cucumis melo*) small RNAome by high-throughput pyrosequencing.** *BMC Genomics* 12: 393." doy mi consentimiento para que éste se presente como parte de los trabajos de la tesis del solicitante D. Daniel González Ibeas. Las actividades que el doctorando ha realizado en el marco de dicho trabajo no se han presentado ni se van a presentar como parte de otra tesis doctoral. Asimismo, declaramos de relevancia la contribución del doctorando en la investigación cuyos resultados se plasman en el citado trabajo.

Madrid, 1 de Marzo de 2012

Firma: Jordi García Mas

Firma: Montserrat Saladié

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A quien pueda concernir:

Como coautor de los trabajos "Gonzalez-Ibeas, Daniel, Joaquin Cañizares, and MA Aranda. *in press*. **Microarray analysis shows that recessive resistance to Watermelon mosaic virus in melon is associated with the induction of defense response genes.** *Molecular Plant-Microbe Interactions* 25 (1): 107-118.", y "Daniel Gonzalez-Ibeas, Jose Blanca, Joaquin Cañizares, Veronica Truniger, and Miguel A. Aranda. 2012. **A cost-effective double-stranded cDNA synthesis for plant microarrays.** *Plant Molecular Biology Reporter*, in press.", doy mi consentimiento para que éste se presente como parte de los trabajos de la tesis del solicitante D. Daniel González Ibeas. Las actividades que el doctorando ha realizado en el marco de dicho trabajo no se han presentado ni se van a presentar como parte de otra tesis doctoral. Asimismo, declaro de relevancia la contribución del doctorando en la investigación cuyos resultados se plasman en el citado trabajo.

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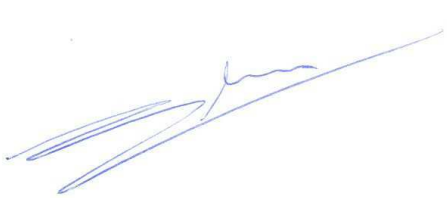

Firma: José Blanca Postigo

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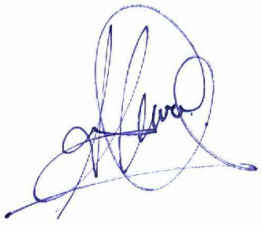
Barcelona, 1 de Marzo de 2012



Firma: Ana Caño Delgado

A quien pueda concernir:

Como coautor del trabajo "Gonzalez-Ibeas, Daniel, José Blanca, Livia Donaire, Montserrat Saladié, Albert Mascarell-Creus, Ana Cano-Delgado, Jordi Garcia-Mas, Cesar Llave, and MA Aranda. 2011. **Analysis of the melon (*Cucumis melo*) small RNAome by high-throughput pyrosequencing.** *BMC Genomics* 12: 393." doy mi consentimiento para que éste se presente como parte de los trabajos de la tesis del solicitante D. Daniel González Ibeas. Las actividades que el doctorando ha realizado en el marco de dicho trabajo no se han presentado ni se van a presentar como parte de otra tesis doctoral. Asimismo, declaro de relevancia la contribución del doctorando en la investigación cuyos resultados se plasman en el citado trabajo.

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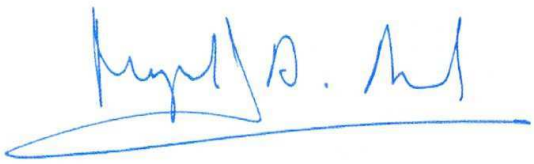
Firma: Albert Mascarell Creus

A quien pueda concernir:

Como coautor/a de los trabajos "Daniel Gonzalez-Ibeas, Jose Blanca, Joaquin Cañizares, Veronica Truniger, and Miguel A. Aranda. 2012. **A cost-effective double-stranded cDNA synthesis for plant microarrays.** *Plant Molecular Biology Reporter*, in press.", "Gonzalez-Ibeas, Daniel, José Blanca, Livia Donaire, Montserrat Saladié, Albert Mascarell-Creus, Ana Cano-Delgado, Jordi Garcia-Mas, Cesar Llave, and MA Aranda. 2011. **Analysis of the melon (Cucumis melo) small RNAome by high-throughput pyrosequencing.** *BMC Genomics* 12: 393." y "Daniel Gonzalez-Ibeas, Jose Blanca, Joaquin Cañizares, Veronica Truniger, and Miguel A. Aranda. 2012. **A cost-effective double-stranded cDNA synthesis for plant microarrays.** *Plant Molecular Biology Reporter*, First Online.", doy mi consentimiento para que éste se presente como parte de los trabajos de la tesis del solicitante D. Daniel González Ibeas. Las actividades que el doctorando ha realizado en el marco de dicho trabajo no se han presentado ni se van a presentar como parte de otra tesis doctoral. Asimismo, declaramos de relevancia la contribución del doctorando en la investigación cuyos resultados se plasman en el citado trabajo.

Murcia, 26 de Marzo de 2012



Firma: Miguel Aranda Regules

A quien pueda concernir:

Como coautor/a del trabajo "Daniel Gonzalez-Ibeas, Jose Blanca, Joaquin Cañizares, Veronica Truniger, and Miguel A. Aranda. 2012. **A cost-effective double-stranded cDNA synthesis for plant microarrays.** *Plant Molecular Biology Reporter*, First Online", doy mi consentimiento para que éste se presente como parte de los trabajos de la tesis del solicitante D. Daniel González Ibeas. Las actividades que el doctorando ha realizado en el marco de dicho trabajo no se han presentado ni se van a presentar como parte de otra tesis doctoral. Asimismo, declaramos de relevancia la contribución del doctorando en la investigación cuyos resultados se plasman en el citado trabajo.

Murcia, 26 de Marzo de 2012

Firma: Verónica Truniger Rietman