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Identification and Characterization of Stress-Responsive *TAS3*-Derived TasiRNAs in Melon

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Small interfering RNAs (siRNA) are key regulators of gene expression that play essential roles in diverse biological processes. *Trans*-acting siRNAs (tasiRNAs) are a class of plant-endogenous siRNAs that lead the cleavage of nonidentical transcripts. TasiRNAs are usually involved in fine-tuning development. However, increasing evidence supports that tasiRNAs may be involved in stress response. Melon is a crop of great economic importance extensively cultivated in semiarid regions frequently exposed to changing environmental conditions that limit its productivity. However, knowledge of the precise role of siRNAs in general, and of tasiRNAs in particular, in regulating the response to adverse environmental conditions is limited. Here, we provide the first comprehensive analysis of computationally inferred melon-tasiRNAs responsive to two biotic (viroid-infection) and abiotic (cold treatment) stress conditions. We identify two *TAS3*-loci encoding to length (*TAS3-L*) and short (*TAS3-S*) transcripts. The *TAS* candidates predicted from small RNA-sequencing data were characterized according to their chromosome localization and expression pattern in response to stress. The functional activity of *cmTAS* genes was validated by transcript quantification and degradome assays of the tasiRNA precursors and their predicted targets. Finally, the functionality of a representative *cmTAS3*-derived tasiRNA (*TAS3-S*) was confirmed by transient assays showing the cleavage of *ARF* target transcripts.

Keywords: Cucurbitaceae • NcRNAs • Plant-environment interactions • Regulation of the stress response in crops • RNA silencing • Small RNAs in melon.

Introduction

Adverse environmental conditions, either of both biotic and/or abiotic origin, cause severe productivity constrains in major crops leading to important economic losses worldwide (Sunkar et al. 2007, Calanca 2017). As sessile organisms, plants have developed various protective mechanisms to counteract stress situations. As a general strategy, gene expression is fine-tuning regulated to assist the physiological changes necessary to ensure plant adaptation to changing environments (Zhang 2015, Zhu 2016, Banerjee et al. 2017, Bielach et al. 2017).

Small regulatory RNAs (21–24 nt in size) are key regulators of gene expression in plants, playing essential roles in diverse biological processes (Bologna and Voinnet 2014, Borges and Martienssen 2015, Martinez and Köhler 2017). Upon production, small RNAs (sRNAs) are loaded into their effector protein named ARGONAUTE (AGO), and guide it to recognize target RNAs or DNA through sequence complementarity (Carbonell 2017, Yu et al. 2017). AGO/sRNA complexes negatively modulate gene expression at both transcriptional and post-transcriptional levels, by directing DNA methylation or histone modification, and through target RNA cleavage or translational inhibition, respectively (D'Ario et al. 2017, Yang et al. 2018).

Endogenous plant sRNAs are classified primarily according to their initial processing source. The small RNAs derived from double-stranded RNA (dsRNA) precursors are known as small interfering RNAs (siRNAs), and sRNAs arising from single-stranded self-complementary hairpin structures are named microRNAs (miRNAs) (Axtell 2013). Although increasing evidences support the notion that miRNAs act as master regulators of the plant responses to environmental changes (Zhang 2015, Sunkar et al. 2012, Kumar 2014, Islam et al. 2018, Sanz-Carbonell et al. 2019), the role of siRNAs in these processes remains obscure.

On the basis of their origin, biogenesis and/or mode of action, endogenous siRNAs can be further classified into phased siRNAs (phasiRNAs), heterochromatic siRNAs (hc-siRNAs) and natural antisense siRNAs (nat-siRNAs) (Axtell 2013, Shriram et al. 2016, Yu et al. 2017).

In plants, transcript targeting by an miRNA can lead in some cases to the production of multiple siRNAs in a phased pattern relative to the miRNA cleavage site. These phased sRNAs are termed phasiRNAs (Fei et al. 2013, Deng et al. 2018). If the miRNA-sliced transcript is a noncoding RNA, it can generate a particular type of phasiRNAs known as *trans*-acting siRNAs (tasiRNAs) because they target in trans-RNAs distinct from those from which they derive (Allen et al. 2005, Axtell 2013, Deng et al. 2018).

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In the tasiRNA biogenesis model, a specific miRNA directs the AGO-dependent cleavage of Pol II-dependent *TAS* transcripts. Next, a protein complex that includes RNA-dependent RNA polymerase 6 (RDR6), suppressor of gene silencing 3 (SGS3) and double-stranded RNA binding 4 (DRB4) (Adenot et al. 2006, Fukunaga and Doudna 2009) is recruited to one of the cleavage fragments to generate a dsRNA. This dsRNA is sequentially processed by DCL4 into 21-nucleotide (nt) tasiRNA duplexes in register with the miRNA-guided cleavage site (Allen et al. 2005, Axtell et al. 2006). One strand of the tasiRNA duplex is selectively loaded into an AGO protein according to the sRNA 5′nt, or to other sequence/structural elements of sRNA, the sRNA duplex or the PIWI domain (Mi et al. 2008, Montgomery et al. 2008, Takeda et al. 2008, Zhu et al. 2011, Zhang et al. 2014).

TasiRNAs were initially identified in *Arabidopsis thaliana* (*Arabidopsis*), where four gene families, *TAS1*, *TAS2*, *TAS3* and *TAS4*, have been described. The processing of *TAS1* and *TAS2* precursors in *Arabidopsis* is initiated by miR173 cleavage, whereas *TAS3* and *TAS4* biogenesis is triggered by miR390 and miR828, respectively (Allen et al. 2005, Howell et al. 2007, Allen and Howell 2010). TasiRNAs have been characterized across a wide range of plant species, from the moss *Physcomitrella patens* (Axtell et al. 2006, Talmor-Neiman et al. 2006, Arif et al. 2012) to higher plants such as rice, maize, pine and tomato (Williams et al. 2005, Axtell et al. 2006, Heisel et al. 2008, Johnson et al. 2009, Wu et al. 2017), suggesting that these regulatory RNAs form part of a common ancient pathway in plants. Although they have been described as regulators of plant developmental processes (Chitwood et al. 2009, Marin et al. 2010, Fei et al. 2013), tasiRNAs may also be involved in abiotic and biotic stress responses (Hsieh et al. 2009, Li et al. 2012, Luo et al 2012, Wu et al. 2017). Among plant *TAS* genes, the most widely studied is *TAS3* whose transcript bears two target sites of miR390, generating tasiRNAs via the so-called 'two-hit' mechanism (Axtell et al. 2006). Very recently, however, it has been shown that—at least in *Arabidopsis*—a single miR390 targeting event is sufficient for *TAS3*-based tasiRNA biogenesis (de Felippes et al. 2017).

Melon (*Cucumis melo*) is a crop of great economic relevance. It is extensively cultivated in semiarid regions frequently exposed to changing environmental conditions that affect its production (Wei et al. 2013). The recent development of molecular tools, such as EST collections (Clepet et al. 2011), TILLING platforms (González et al. 2011), and genome sequencing and annotation (Garcia-Mas et al. 2012, Ruggieri et al. 2018), has favored the emergence of melon as a valuable experimental system to conduct significant agricultural-related research focused on development (Wu et al. 2018) or response to stress (Bustamante et al. 2018, Sanz-Carbonell et al. 2019). Although a melon genome region containing two predicted miR390 target sites able to generate phased sRNAs and reminiscent to *TAS3* genes was previously identified computationally (Gonzalez-Ibeas et al. 2011), the role that siRNAs in general and tasiRNAs in particular play in the regulation of the response to stress in melon plants remains in a conundrum.

In order to gain insight into stress-responsive *TAS*-derived tasiRNA in melon, potential TAS loci (*CmTAS*) were computationally inferred. Next, we located these *cmTAS* in the melon chromosome and analyzed their expression patterns in response to stress. Finally, we functionally validated *CmTAS* genes by analysis of melon degradome and transient assays in *Nicotiana benthamiana*. The results shown here indicate that the processing of *TAS3*-derived tasiRNAs in melon is a functional process related to environmental conditions.

Results

Prediction and annotation of melon TAS loci

For genome-wide detection of putative *TAS* loci in melon, the *TASI-PREDICTION* tool included in the *UEA small RNA Workbench* (v.4.4) was employed using as input data the reads recovered from sRNA libraries obtained from melon plants exposed to seven biotic and abiotic stress conditions (Bustamante et al. 2018, Sanz-Carbonell et al. 2019) and the melon genome sequence (Version 3.5.1). The software parameters established for a more robust prediction of phased siRNAs in melon are detailed in the Materials and Methods section. In all, 895 regions were identified as being potential phasi-generators loci in the melon genome (Supplementary Table S1).

Then these potential *TAS* precursors were filtered according to the following parameters: (i) homology with known *TAS* loci and (ii) $\geq$5 phased sequences identified (at least one in both senses).

We identified four melon genomic regions highly homologous to known *TAS* genes: one (*TAS-Cmel779*) matching *Arabidopsis TAS2* and three (*TAS-Cmel028*, *TAS-Cmel735* and *TAS-Cmel737*) homologous to *TAS3* precursors identified in *Wolffia arrhiza*, *Solanum demissum* and *Oryza sativa*, respectively (Supplementary Table S2). Two of the potential *TAS* precursors (*TAS-Cmel779* and *TAS-Cmel028*) were discarded because of the low number (three) of phased sequences recovered and the shortness (32 nt) of the homologous region, respectively. The two selected potential *TAS3* loci (*TAS-Cmel735* and *TAS-Cmel737*) were localized in chromosome 11. Regarding the number of phased sRNAs matching predicted *TAS* regions, *TAS-Cmel735* with 23 sequences (12 sense and 11 antisense) was the most highly represented.

To broaden our search, potential phasiRNA precursors nonhomologous to known *TAS* genes were reanalyzed following a strategy similar to that previously described to identify phased siRNAs in grapevine (Zhang et al. 2012). First, we retrieved a sequence including the genomic region containing the predicted *TAS* flanked by 100 nt. Next, we searched for predicted regions between melon miRNAs and the retrieved genomic sequence using psRNATarget. As a final step, we validated the predicted miRNA cleavage position analyzing our degradome dataset. Under this analysis condition, we identified an additional potential *TAS* loci identified as *TAS-Cmel087* sliced in its 5′ region by miR828, reminiscent to *TAS4* in *Arabidopsis*. However, this predicted precursor was discarded because of the low number (four) of phased sequences

recovered (Supplementary Table S2). The process employed to identify potential *TAS* loci in melon is represented in Supplementary Fig. S1.

The actual sequences of both, *Cmel735* and *Cmel737*, predicted *TAS* loci recovered from the melon genome were confirmed by the sequencing of PCR-amplified products (Supplementary Fig. S2A). Finally, the presence of transcripts derived from these predicted *TAS* loci was validated by RT-PCR assays and the subsequent sequencing of the amplified products (Supplementary Fig. S2B).

Identification and validation of potential tasiRNA triggers

Considering that the 'two-hit' mechanism (Axtell et al. 2006) is the commonest processing mechanism to trigger *TAS3* derived tasiRNAs in plants, as last filtering criteria we considered as potential *TAS* precursors only those containing the double recognition sites for the melon miRNAs found in the miRbase. To identify miRNA target sites in these potential *TAS* precursors, miRNA binding site prediction was performed using the *psRNAtarget* server (Dai et al. 2018). As shown in Supplementary Table S2, two miR390 target sites for melon miRNAs recovered from the miRBase were predicted for both *TAS-Cmel735* and *TAS-Cmel737*. As expected (the functional complex *TAS3*/miR390 is highly conserved in plants) (Xia et al. 2017), the two predicted *cmTAS3* precursors included miR390 target sites. For *TAS-Cmel735* and *TAS-Cmel737*, the miR390 target sites were located between the positions 24043012–24042992 (cut at 5′) and 24042821–24042801 (cut at 3′), and between positions 28041083–28041103 (5′) and 28041335–28041355 (3′) of chromosome 11, respectively (Supplementary Table S2). *TAS-Cmel 735* (212 nt) was relatively short compared with the *TAS-Cmel 737* (273 nt), resembling the previously observed in apple and strawberry when two *TAS3* families encoding length (*TAS3-L*) and short (*TAS3-S*) transcripts, respectively, were described (Xia et al. 2012, Xia et al. 2015).

Predicted miRNA-mediated processing of *TAS-Cmel735* and *TAS-Cmel737* precursors was evaluated by 5′-RLM-RACE assays. The results indicated that only remnants derived from the canonical (cleavage position between nucleotides 10 and 12 in relation to the 5′-end of the miRNAs) cut of *TAS-Cmel735* guided by miR390 in both 5′ and 3′ regions were recovered from our dataset (**Fig. 1;** Supplementary Fig. S3A, B). According to the number of normalized reads recovered from degradome assay matching onto both 5′ and 3′ regions [2 reads per million (RPM) and 2,734 RPM, respectively], it was possible to infer that the 3′ region was the most highly processed and could possibly act as the starting point for the sequential processing of *TAS-Cmel735* (potential *TAS3*) in melon. As expected, when the 21 nt-long sRNAs recovered from the melon sRNAs libraries were plotted onto the potential *TAS-Cmel735* genomic region, all analyzed sequences matched exclusively the area comprised within both miR390 target sites, which according to the biogenesis model for tasiRNA-production, constitutes the external limits of the miRNA-processed *TAS* precursor (**Fig. 2A**). In coincidence with the previously described for *TAS3-S* family in other dicotyledonous species (Xia et al. 2015), the residual miR390-based slicing of the *TAS* precursor in its 5′ region was able to trigger the production of phased siRNAs (**Fig. 2C**). The observation that poor *TAS3-S* processing at its 5′ end is sufficient to trigger high accumulation of phased siRNAs suggests that there is no direct correlation between the efficiency of miR390-mediated slicing and the extent of subsequent phased-siRNA production. In addition, and contrarily to the expected, when a similar analysis was performed onto the *TAS-Cmel737* transcript (that according our degradome data are not sliced by miR390), all analyzed sRNA sequences matched exclusively the area comprised within both computationally inferred miR390 target sites and exhibit high accumulation of miR390-related phased siRNAs (Supplementary Fig. S3C).

Determination of *TAS3*-derived tasiRNAs targets in melon

To elucidate the basis of the regulatory pathways modulated by the predicted *cmTAS3*, we analyzed the existing relationship between the phased siRNAs recovered from our dataset and their intended targets. According to the miR390-guided cleavage site validated by 5′-RLM-RACE in the *TAS-Cmel735*, nine potential 21-nt-long tasiRNAs were predicted to be produced from the processed *TAS3* precursor in melon (**Fig. 2B**). These in-phase siRNAs were named 5′D1[+] to 5′D9[+], starting from the miR390 cleavable target site identified in the 3′ region of the potential *TAS* transcript. In addition, we also analyzed potential phased siRNAs arising from the unprocessed miR390 target position predicted in the transcripts derived from *TAS-Cmel737* (Supplementary Fig. S3D).

On the basis of psRNA-target results (considering only transcripts with well-established biological function), the lower expectative values (expect. $\leq$1) were obtained for three melon transcripts that were predicted as candidates to be negatively regulated by *TAS-Cmel735*-derived tasiRNA 5′D6[+] and *TAS-Cmel737*-derived tasiRNA 5′D7[+] and 5′D8[+] (Supplementary Table S3). Consistently with the tasiRNA targets established in *Arabidopsis* (Xia et al. 2017), mRNAs related to AUXIN RESPONSE FACTORS (ARF) family (ARF2, ARF3 and ARF4) were identified as potential targets for *TAS3*-derived siRNAs (tasiARF) activity. As observed in diverse plant species, two recognition sites for tasiARF-AGO activity were identified in *ARF* transcripts (Supplementary Table S3). Like the commonly described for *TAS3-L* family, two tasiARFs were derived from the *TAS-Cmel737* transcript. In contrast, *TAS-Cmel735* transcript encodes only one tasiARF, sharing also structural resemblance with *TAS3-S* family (Xia et al. 2012, Xia et al. 2017).

To validate the functionality of the predicted tasiRNA-target interactions, we analyzed by 5′-RLM-RACE the processing of the transcripts expected to be regulated by tasiARF in melon. As shown in **Fig. 3**, for the ARF3 melon transcript, we detected sequences-remnants coincident with those expected for transcripts sliced via *TAS3*-derived tasiRNA-guided AGO activity in

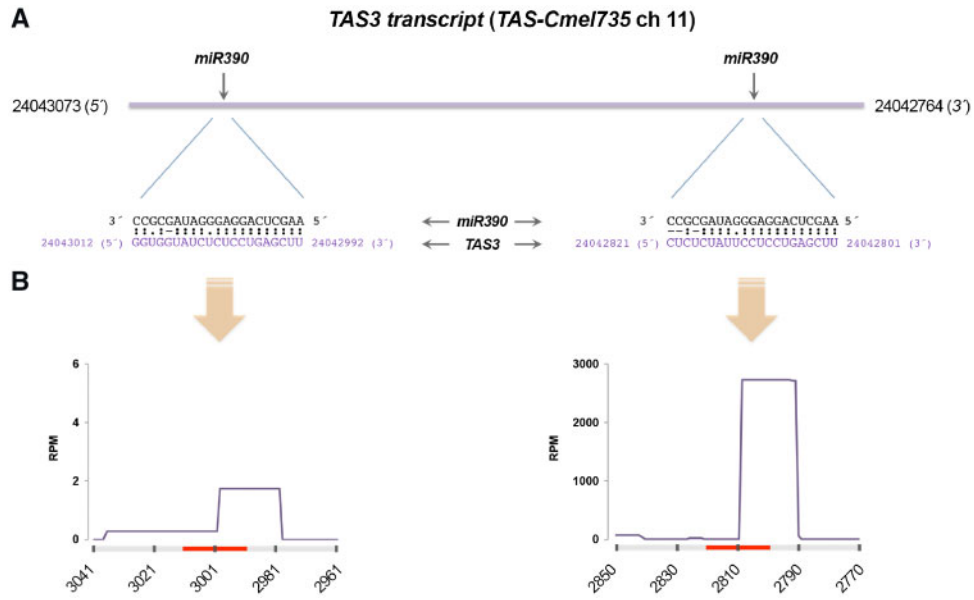

Fig. 1 The predicted melon *TAS3-S* transcript is sliced by miR390. (A) Graphic representation (not to scale) of the potential *TAS3-S* transcript (*TAS-Cmel735*) identified in the melon genome. The region position in chromosome 11 is also detailed. The miR390 target sites predicted by *psRNA-targets* tool are marked with gray arrows. miR390 sequence is shown in gray. The complementary sequence identified in *TAS3* transcripts is denoted magenta. (B) Graphic representation of the miR390-cleaved *TAS3-S* transcripts detected by high-scale degradome assay. The obtained sequences were plotted (allowing 100% homologous matching) onto the *TAS3* sequence. The red lines on the *X*-axis indicate the position of the predicted miRNA recognition site in the melon *TAS3* transcript. The values on the *Y*-axis represent the number of obtained reads (normalized in RPM).

the two predicted target sites. In contrast, only one of the two cleavage positions estimated by psRNA target for the tasiRNA-ARF4 interaction was validated by the degradome assays (Fig. 3). Finally, sequence-remnants coincident with the expected processing of ARF2 by *TAS-Cmel737* derived tasiRNA 5′D7[+] and 5′D8[+] were also detected (Fig. 3).

In vivo validation of the interplay tasiARF/target

To provide further evidence for the biological activity of the *TAS3*-derived tasiARF identified in melon, the tasiARF derived from *TAS-Cmel735* transcript (*TAS3-S*) was expressed from a functional syn-tasiRNA vector including a modified *TAS1c* precursor from *Arabidopsis* (Carbonell et al. 2014). The resulting syn-tas-cm*TAS3* construct is shown in Fig. 4A. As a functional reporter of tasiRNA activity, we used a construct containing a common region of ARF3 and ARF4 cDNA consisting of the predicted tasiARF target site (AGGUCUUGCAAGGUCAAG AAA) fused to the 3′ end of the yellow fluorescent protein (YFP) mRNA. The detailed design of the YFP-ARF reporter construct is shown in Fig. 4B.

Constructs syn-tas-cm*TAS3* and ARF-YFP, plus a construct expressing miR173 needed for triggering *TAS1c* processing (Montgomery et al. 2008), were cloned in *Agrobacterium tumefaciens* and coinfiltrated in *N. benthamiana* leaves for transient expression analysis. The correct processing and accumulation of the *syn-tas*-cm*TAS3* were validated by stem-loop quantitative RT-PCR (qRT-PCR; Supplementary Fig. S4A). A construct expressing an amiRNA targeting *GUS* transcripts (Carbonell and Daros 2017) was used as negative control. Infiltrated leaves were analyzed at 2 days post infiltration (2 dpi) by confocal microscopy. The expression of the YFP-ARF reporter was suppressed in the leaves coinfiltrated with the syn-tasiRNA3 construct, whereas the leaves coinfiltrated with the control construct targeting GUS transcripts showed typical fluorescence (Fig. 4C). Based on our qRT-PCR analysis, the decrease of ARF-YFP transcript accumulation in silenced plants reinforces the results obtained by confocal microscopy (Supplementary Fig. S4B). These results suggest that lack of expression of the YFP-ARF reporter coinfiltrated with the syn-tasiRNA3 construct is due to the tasiRNA-mediated degradation of its mRNA, hence validating in vivo the functional activity of tasiARF as negative regulator of ARF3 and ARF4 transcripts in melon.

Functional activity of stress-responsive tasiRNAs

To gain mechanistic insights into the regulatory role of tasiARF in response to stress, we analyzed the accumulation of the target transcripts in those stress situations in which *TAS3*-derived tasiRNAs (tasiARF) showed significant differential accumulation in response to stress.

To evaluate the effects of changing environments on tasiARF populations, we used our data previously obtained from pairwise comparisons of total sRNAs recovered from control and treated samples with the statistical testing method DESeq2 (Sanz-Carbonell et al. 2019). According to our established filtering criteria (log2FC ≥ 1 or ≤ -1, FDR value <0.05, and base mean ≥ 5), tasiARF accumulation was significantly reduced in the melon plants exposed to cold treatment (for both *TAS3-L*

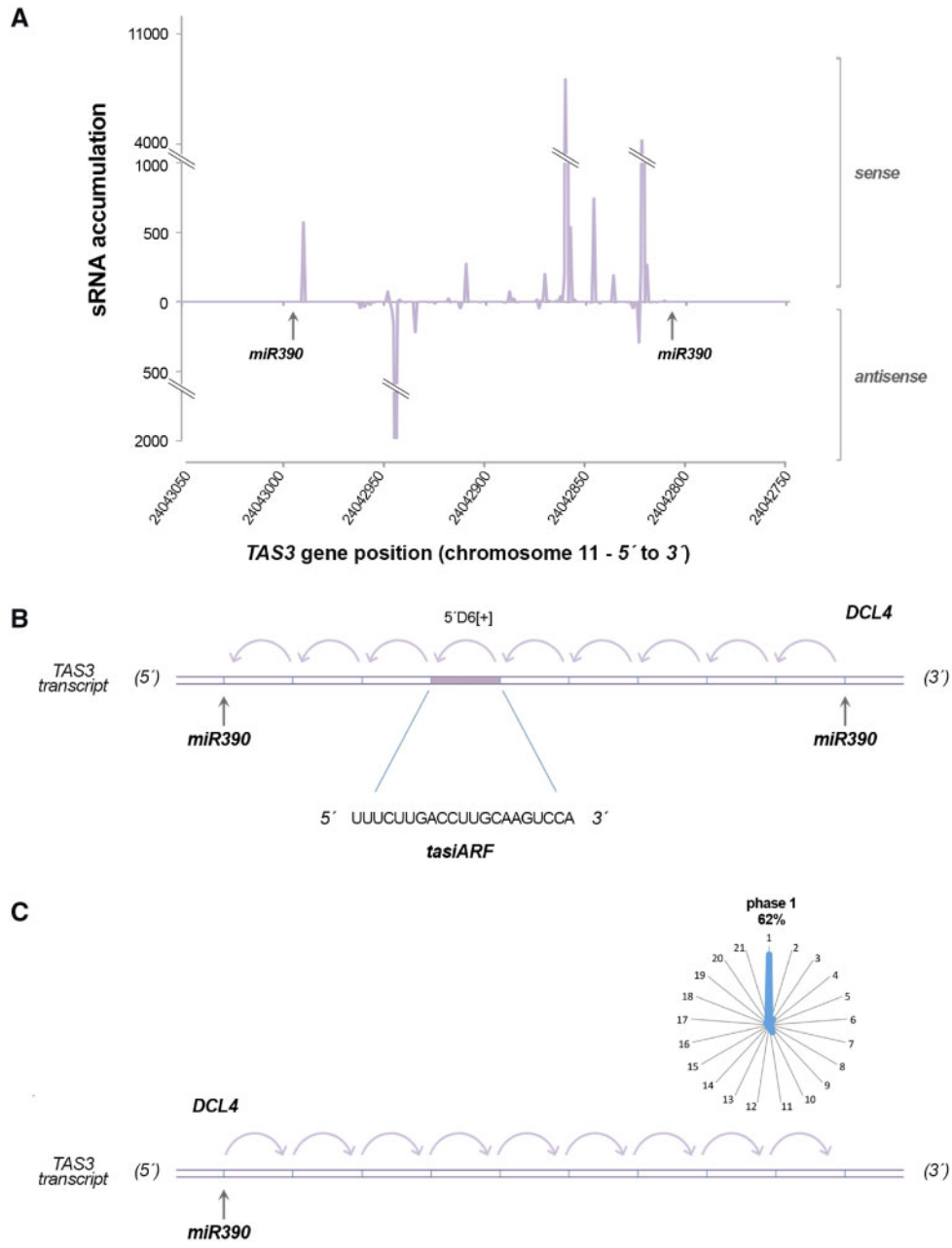

Fig. 2 Phased tasiRNAs arising from the melon *TAS3* transcripts. (A) Read abundance distribution of *TAS3*-derived sRNAs in melon. Twenty-one nucleotides in length sRNAs (recovered from control libraries) were plotted (allowing 100% homologous matching) onto *TAS3* region in melon genome. Arrows indicate miR390 target sites. The *X*-axis represents a double-stranded *PHAS* locus. The reads abundance is represented in RPM. (B) Schematic representation of the processing inferred for the *TAS3*-S precursor in melon. miR390 target sites in the 3′ region are marked. Phased 21 nt in length *TAS3-S* derived tasiRNAs predicted computationally and recovered by sequencing are represented. The position of the tasiRNA identified as a negative modulator of ARF transcripts (tasiARF) is highlighted at the position 5′D6[+] and its sequence detailed. (C) Phased 21 nt in length derived from the miR390-guided processing it the 5′ region of the *TAS3-S* transcript predicted computationally and recovered by sequencing are represented. Radar plots show percentages of 21-nt reads corresponding to each one of the 21 registers from *TAS3-S* transcripts, with position 1 designated as immediately after the miR390-guided predicted cleavage site in the 5′ region.

and *TAS3-S* derived tasiRNAs) and HSVd infection (for *TAS3-S* derived tasiRNA) (**Table 1;** Supplementary Table S4). These results revealed that, in coincidence with the observed in other plant species (Moldovan et al. 2010, Katiyar et al. 2015, He et al. 2018), the biogenesis of *TAS3*-derived tasiRNAs is a process susceptible to be altered by adverse environmental conditions. qRT-PCR assays revealed that the accumulation of ARF2, ARF3 and ARF4 transcripts is significantly increased in cold-treated and HSVd-infected plants, as expected for the functional negative regulation of the *TAS3*-derived tasiARFs, whose accumulation decreases in the same conditions, on its predicted targets. (**Fig. 5**).

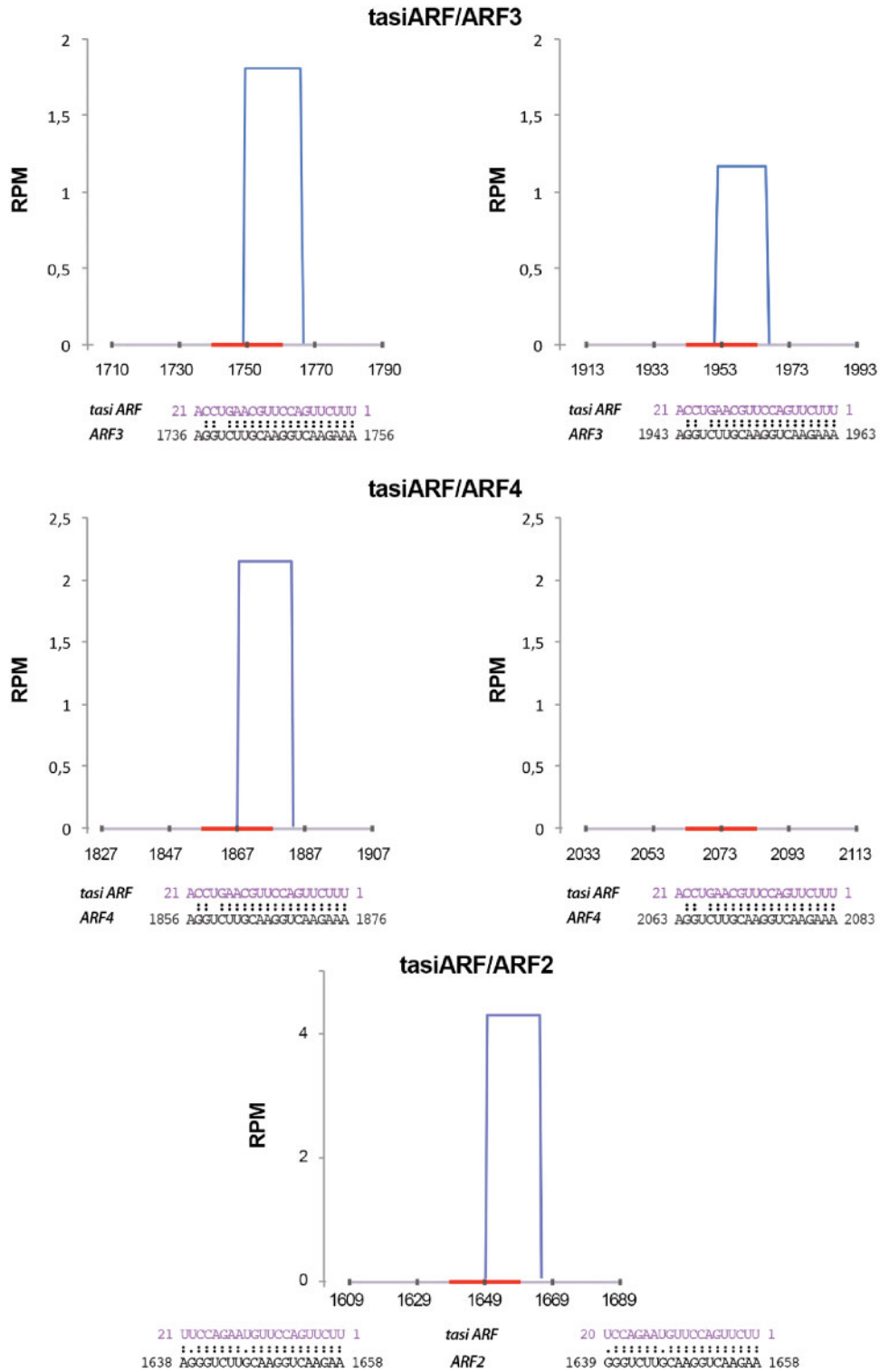

Fig. 3 ARF family members are recognized as targets for *TAS3* tasiRNA activity in melon. Graphic representation of tasiRNA-cleaved *ARF3*, *ARF4* and *ARF2* melon transcripts detected by degradome assay. The obtained sequences were plotted (allowing 100% homologous matching) onto the *ARF3* (XM017043883.1), *ARF4* (XM008465701) and *ARF2* (XM-008466144) sequences. The red lines on the *X*-axis show the position of the predicted tasiRNA target sites. The values on the *Y*-axis represent the number of obtained reads (normalized in RPM). The tasiARF sequence is denoted in magenta. The complementary sequences identified in target transcripts are presented in gray.

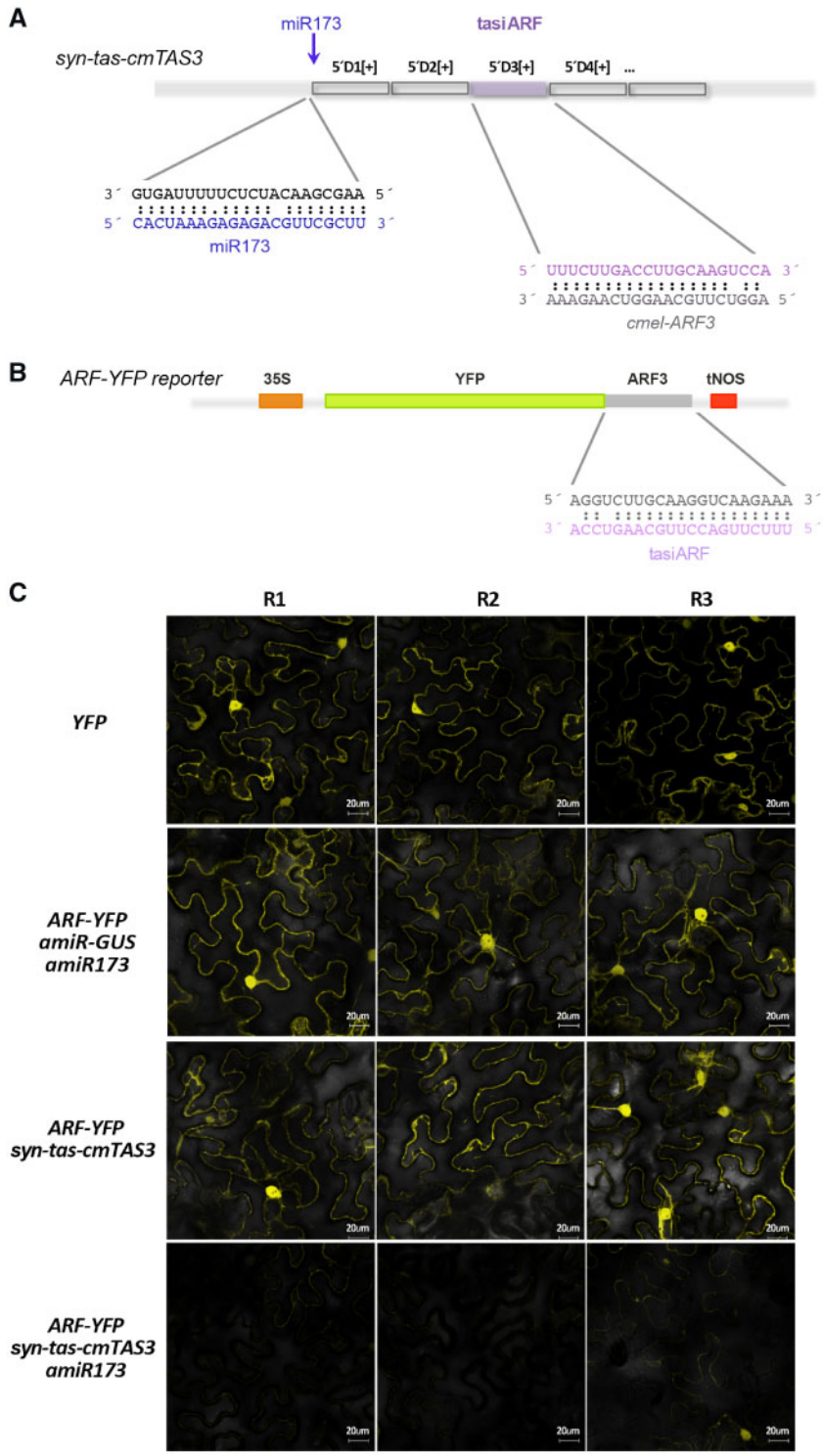

Fig. 4 The *TAS3*-derived tasiARF trigger AGO-mediated processing of target transcripts in vivo. (A, B) Physical map (not to scale) of the constructs used herein. The miR173 and tasiARF target sites are detailed. miR173 and tasi-ARF sequences are denoted in blue and magenta, respectively. The Cauliflower mosaic virus 35S promoter and the nopaline synthase terminator (t-Nos) are also represented. (C) *Nicotiana benthamiana* plants were coinfiltrated with agrobacterium transformed with (i) YFP-ARF reporter plus amiR-GUS and amiR173 and (ii) YFP-ARF reporter plus syn-tas-cmTAS3 as control. As observed in the lower panels, the YFP-ARF reporter expression is clearly reduced compared with control (middle panels), when is coexpressed with the syn-tasiRNA and the amiR173 constructs. The unmodified YFP (upper panels) was used as YFP expression control. R1 to R3 are replicates of the same experiments in different *N. benthamiana* plants.

Discussion

SiRNAs have been shown to play important roles in developmental regulation and stress responses in plants. Genome-wide identification of diverse biogenesis-grouped members of the 'siRNA family' in an increasing number of plant species may have a high impact in the investigation of the molecular basis of gene regulation in sRNA-mediated stress response (Banerjee et al. 2017, Li et al. 2017, Kumar et al. 2018). In this study, we used a computational approach to identify putative *TAS* loci in the melon genome. As a consequence of a strict selective process, only two potential *TAS3* loci (*TAS-Cmel735* and *TAS-Cmel737)* were considered as robustly predicted and subjected to subsequent validation analysis.

To date, there are two families of *TAS3* loci described in plants, which are identified as *TAS3*-short (*TAS3-S*) and TAS3-long (*TAS3-L*), respectively (Xia et al. 2017). In *TAS3-L*, only the 3′ miR390 target site is cleavable, generating two in-phase tasiARFs (Allen et al. 2005, Axtell et al. 2006). The 5′ target site of *TAS3-L* is usually noncleavable (Axtell et al. 2006). In contrast, both miR390 target sites of *TAS3-S* are cleavable, and one unique tasiARF is generated. Interestingly, has been described that both 5′ and 3′ processed ends can potentially generate phased siRNAs (Xia et al. 2015, Xia et al. 2017).

Our results evidence that, in coincidence with the previously described in apple (Xia et al. 2012) and strawberry (Xia et al. 2015), melon plants possess both *TAS3-L and TAS3-S* families that transcribe long and short transcripts, respectively. Although the *TAS3-S* family is not present in *Arabidopsis*, it is conserved in many other dicot species (Xia et al. 2015). The existence of *TAS3*-derived transcripts in melon was initially validated by RT-PCR amplification. The prediction of well-established miR390-target sites at two (at both 5′and 3′ region) positions of the *TAS3*-derived transcript and the precise determination of such AGO-mediated processing by degradome assays provided the first experimental evidences supporting the presence of a 'bona fide' *TAS3-S* loci in melon. miRNA-mediated cleavage is considered to be an important and necessary aspect of tasiRNA biogenesis, not only for ensuring sRNA production in the proper register, but also to recruit RDR6 and SGS3 to the ncRNA transcript. Surprisingly, we were incapable to confirm by degradome assay the miR390-mediated processing of the 3′ region of the *TAS3-L*. However, the recovering of high levels of phased siRNAs derived from the longer family member suggests that, in melon, tasiARFs might be generated from a 3′ noncleavable *TAS3-L* transcript. Although we cannot exclude the possibility that sequences-remnants derived from the miR390-mediated processing of the *TAS3-L* transcripts were under-represented in our degradome dataset, this result is coincident with the recently described in *Arabidopsis* where a single noncleavable miRNA hit was sufficient requirement for RDR6 recruitment and functional tasiRNA biogenesis (Arribas-Hernández et al. 2016, de Felippes et al. 2017).

Table 1 Detail of the expression values (estimated by DeSEQ analysis) of the *TAS3*-S derived tasiARF sequence in melon plants exposed to diverse biotic and abiotic stress conditions

tasiRNA	Stress	Base mean	Log_2FC	FDR
TTTCTTGACCTTGCAAGTCCA	Drought	16.053	−0.677	0.589
TTTCTTGACCTTGCAAGTCCA	HSVd	15.671	−1.762	0.012
TTTCTTGACCTTGCAAGTCCA	Cold	16.961	−1.667	0.027
TTTCTTGACCTTGCAAGTCCA	Salinity	18.479	−0.438	0.760
TTTCTTGACCTTGCAAGTCCA	Mon	21.531	0.036	0.999
TTTCTTGACCTTGCAAGTCCA	Agro	15.630	−0.788	0.694
TTTCTTGACCTTGCAAGTCCA	Short day	29.119	0.888	0.368

Only LogFC values ≥ 1 or ≤ -1 and FDR values <0.05 were considered as significant for 'bona fide' stress-responsive tasiRNAs.

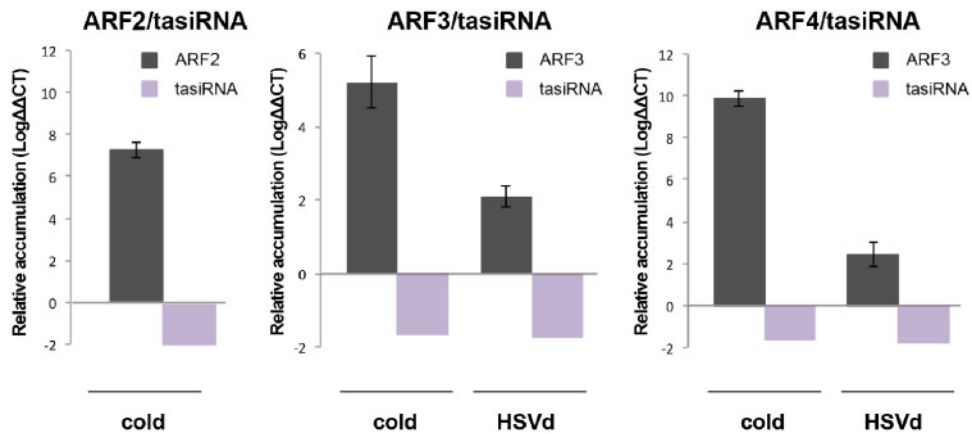

Fig. 5 The ARF2, ARF3 and ARF4 expression correlate to the changes in the accumulation of *TAS3*-derived tasiRNAs responsive to stress conditions. (A) Histogram showing the means of the relative accumulation respect to the control samples (in Log of ΔΔCT value) of ARF3, ARF4 and ARF2 transcripts (gray bars) in melon plants exposed to cold treatment and HSVd infection (ARF3 and ARF4) and cold treatment (ARF2) as estimated by qRT-PCR. Error bars show the confidence interval of the difference between means. The bars in magenta represent the accumulation levels (estimated by sRNA-sequencing data analysis) (in LogFC) of the *TAS3*-derived tasiRNA in the correspondent melon samples (detailed data in **Table1;** Supplementary Table S4). In *TAS3-L* derived tasiRNA is represented the LFC value obtained for the 5? D8 sequence.

It is well established that the miR390/TAS3-ARF pathway represents a highly conserved negative regulator of the mRNAs encoding AUXIN RESPONSE FACTORS (ARF) in plants (Xia et al. 2017). Consequently, the prediction (by psRNA target) and validation (by degradome assays) that *ARF2*, *ARF3* and *ARF4* transcripts in melon are targets for the *TAS3*-derived tasiRNAs (tasiARF), contributed to reinforce our initial prediction. Finally, the demonstration that, in heterologous transient expression assays, the *TAS3-S* tasiRNA is able to negatively regulate 'in vivo' the expression of a target-containing transcript, provides robust evidence supporting the regulatory role of the first *TAS* locus identified in melon.

Regarding its behavior in response to stress conditions, we observed a significant reduction of *TAS3*-derived tasiARF in response to cold treatment (for both *TAS3-L* and *TAS3-S* derived tasiRNAs) and Hop stunt viroid (HSVd) infection (for *TAS3-S* derived tasiRNA). Hence, these results suggest that the biogenesis of *TAS3*-derived tasiRNAs in melon plants is a dynamic process susceptible to be influenced by environmental conditions. The recent description (Sanz-Carbonell et al. 2019) of the decreased accumulation of miR390 (the key component responsible for triggering *TAS3* processing) in the melon plants exposed to both cold and HSVd treatments provides further functional support to this notion.

In line with a more general viewpoint, the incidence of environmental cues in the *TAS3* processing reported herein agrees with previous results that have indicated a close interrelation between tasiARF accumulation and stress conditions in poplar (He et al. 2018), *Arabidopsis* (Moldovan et al. 2010) and sorghum (Katiyar et al. 2015). Interestingly, the observation that the general tendency of the alterations at the tasiARF level (downregulation) observed in melon plants exposed to cold and HSVd was contradictory to that described for the *TAS3* derived tasiRNAs in poplar plants exposed to saline conditions (where upregulation of *TAS3*-derived tasiRNAs was reported). This suggests that similarly to that described for stress-responsive miRNAs (Kumar 2014), changes in the accumulation level of stress-responsive tasiRNAs might be stress- and/or specie-dependent.

As mentioned above, *TAS3*-derived tasiRNAs mediate the cleavage of the transcripts encoding ARFs factors. These proteins are key components of the auxin-signaling cascade and directly control the transcription of primary auxin-responsive genes (Guilfoyle and Hagen 2007). The miR390/TAS3/ARFs module is functionally diverse and regulates multiple plant developmental processes, including leaf patterning and expansion, phase transition, initiation of the shoot meristem, and also plays an important role in root nodule symbiosis (Adenot et al. 2006, Fahlgren et al. 2006, Garcia et al. 2006, Marin et al. 2010, De Luis et al. 2012, Yifhar et al. 2012, Zhou et al. 2013, Li et al. 2014, Cabrera et al. 2016). However, recent studies have revealed that *TAS3*-regulated ARFs are involved in responses to adverse environmental conditions. For instance, it has been described that the miR390/*TAS3*/ARFs module is a key regulator of root growth in poplar subjected to salt stress via the modulation of the auxin pathway (He et al. 2018). In rice, *ARF16* has been related to phosphate starvation responses (Shen et al. 2013), whereas *ARF12* has been associated with phosphate homeostasis. *ARF* genes responsive to changes in water levels have been described in soybean (Wang et al. 2014). Finally, differential expression of *ARF* transcripts in response to diverse abiotic stress conditions was also reported in tea (Xu et al. 2016) and banana plants (Hu et al. 2015). However, the regulatory pathways underlying this phenomenon remain in a conundrum (He et al. 2018).

Given that in general tasiRNA-defective mutants exhibit accelerated vegetative phase-change phenotypes, it has been suggested that *Arabidopsis TAS3*-derived tasiRNAs could be involved in the regulation (mediated by ARF) of developmental processes in leaves and flowers (Guilfoyle and Hagen 2007). Considering that equivalent pathways might be regulated by ARFs in melon, it is reasonable to assume that the downregulation of the *TAS3*-derived tasiRNA observed in cold-exposed and HSVd-infected plants might be related to the developmental alterations observed in plants growing under these adverse environmental conditions, mainly characterized by delayed growth (Hataya et al. 2017, Hou et al. 2018). However, we cannot exclude the possibility that changes in the ARF levels observed in cold exposed and HSVd-infected plants may be also modulated, at least in part, by and stress-related increasing in its transcription level, resembling the previously observed in diverse plant species such as rice (Jain and Khurana 2009), sorghum (Wang et al. 2010), banana (Hu et al. 2015) and pepper (Yu et al. 2017). Further functional evidence, which is difficult to obtain in melon, is necessary to experimentally support this hypothesis. Altogether, our results support that in coincidence with the previously described in *Arabidopsis* (Hsieh et al. 2009, Luo et al. 2012) and tomato (Wu et al. 2017), tasiRNAs could emerge as potential modulators of the plant–environment interactions in melon plants.

Materials and Methods

Sequence data

The sRNA sequence data used herein were obtained from 24 (three replicates by each treatment) previously described (Sanz-Carbonell et al. 2019). cDNA libraries obtained for control and stress-treated—(i) abiotic agents: cold, drought, salinity, short day; (ii) biotic agents: Hop stunt viroid (HSVd), *Monosporascus cannonballus and A. tumefaciens*—melon plants (Supplementary Table S5). The sequence data are publicly available in the SRA genomic repository of NCBI (BioProject IDPRJNA491809).

The statistical testing method DESeq2 v.1.18.1 (Love et al. 2014, https://bioconductor.org/packages/release/bioc/html/DESeq2.html) was used to evaluate the differential expression of the siRNAs in melon plants under stress conditions. Only the sRNAs with: (i) $\log_2$-fold change ($\log_2$FC) ≥ 1 or ≤ -1 for biological significance, (ii) FDR value <0.05 and (iii) base mean ≥ 5, which is the mean of normalized counts of all samples were considered as differentially expressed.

Identification of TAS candidate genes

In order to detect phased tasiRNAs in melon, the *TASI-PREDICTION* tool (http://srna-workbench.cmp.uea.ac.uk/tasi-prediction) was used with default parameters on previously described sRNA libraries obtained from melon plants (Sanz-Carbonell et al. 2019) and from melon genome sequences (Version 3.5.1) downloaded from https://www.melonomics.net. Predicted *TAS* regions were then aligned against known *TAS* sequences described for

Viridiplantae in the NCBI database (https://www.ncbi.nlm.nih.gov) by command-line interface (Rstudio) using BLAST+ (https://www.ncbi.nlm.nih.gov/guide/howto/run-blast-local). Only sequences with homology levels ≥70% and minimum length alignment of 30 nt were considered as potential *TAS* precursors. The sequence corresponding to the *TAS3* gene identified in melon was deposited in the NCBI gene database with the accession number MK410640.

qRT-PCR assays

Total RNA was extracted from pooled leaves (~0.1 g) recovered from the treated and control melon plants using the TRI reagent (SIGMA – ALDRICH, San Luis, MO, USA) according to the manufacturer's instructions. To analyze target expression, total RNA (1.5 μg) extracted from control or treated plants was subjected to DNase treatment (EN0525, Thermo Scientific™) followed by reverse transcription using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific™) according to the manufacturer's instructions by using of oligo-dT. Then, real-time PCR was performed (by triplicate) as previously described (Bustamante et al. 2018). The efficiency of PCR amplification was derived from a standard curve generated by four 5-fold serial dilution points of cDNA mixed from the two samples. Relative RNA expression was determined by using the comparative ΔΔCT method (Livak and Schmittgen 2001) and normalized to the geometric mean of PROFILIN (NM001297545.1) and ADP-ribosylation factor-like (XM_008463181.2) expression, as reference control. Quantification of *syn-tas-cmTAS3* and melon miRNAs was performed starting from low-molecular-weight RNA (<200 nt) fractions obtained as described previously (Sanz-Carbonell et al. 2019). A slightly modified stem-loop-specific reverse transcription protocol for miRNA detection (Czimmerer et al. 2013) was performed as previously described (Sanz-Carbonell et al. 2019). Primers used are listed in Supplementary Table S6.

Degradome assay

Data of AGO-processed sequences used in this work were recovered from degradome libraries obtained from melon plants (Sanz-Carbonell et al. 2019).

Cloning and vectors construction

The syn-tasiRNA construct syn-tas-cm*TAS3* was generated by annealing oligos AC-154 (ATTATCTTGACCTTGTAAGACCCAA) and AC-155 (GTTCTTGGGTC TTACAAGGTCAAGA), obtained with the P-SAMS website tool (Fahlgren et al. 2016), and by ligating the resultant insert into *pMDC32B-AtTAS1c-B/c* (Addgene, Watertown, MA, USA) as previously described (Carbonell et al. 2014). Constructs amiR-GUS and miR173 have been generated and used in previous works (Montgomery et al. 2008, Carbonell and Daros 2017).

The ARF-YFP reporter is constituted by an ARF sequence (derived from both ARF3 and ARF4) predicted as target for the *TAS3*-derived tasiRNA (tasiARF), fused to the 3′ end of the sequence encoding for the YFP and cloned in a binary plasmid pMOG800 under the control of the Cauliflower mosaic virus 35S promoter and the *A. tumefaciens* nopaline synthase terminator (t-Nos) (Knoester et al. 1998). This construct was generated by amplifying the YFP-cDNA with a forward primer that includes an *Nco*I recognition site (GG TCTCCCATGGATGGTGAGCAAGGGCGA) and a reverse primer that contains the ARF sequence and an NheI recognition site (GGTCTCGCTAGCTCTTGACC TTGCAAGACCTTATCACTTGTACAGCTCGCC). The PCR-amplified DNA was digested with NcoI and NheI and ligated into a linearized pMOG800 vector. A nonmodified 35S-YFP-tNos construct was used as agro-infiltration-control.

Agro-infiltration assays

Nicotiana benthamiana leaves of 3- to 4-week-old plants were agroinfiltrated with the corresponding cultures of *A. tumefaciens* strain C58C1, previously transformed with the construct to be analyzed. The overnight grown bacterial culture was diluted in infiltration buffer (0.1 M MES, 1 M $MgCl_2$) up to an optical density at 600 nm (OD600) of 0.2 and injected on the abaxial side of the leaves using a 1-ml needle-less syringe (Gómez and Palls 2007). Agroinfiltrated plants were analyzed 2 days post-agroinfiltration. The growing conditions were as follows: photoperiod of 16 h under visible light (wavelength between 400 and 700 nm) with an irradiance of 65–85 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and 8 h of darkness; temperature cycles of 25°C (light) and 18°C (darkness) and relative humidity of 60–65% (light) and 95–100% (darkness). YFP fluorescence was observed using an inverted Zeiss LSM 780 confocal microscope and ZEN software (Carl Zeiss, Oberkochen, Germany). Leaf dishes were cut and mounted in water. Then, images were acquired using an objective plan-apochromat 40×/1.4 Oil DIC M27 (0.5cm diameter).

Supplementary Data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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