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Salicylic acid and gentisic acid induce RNA silencing-related genes and plant resistance to RNA pathogens

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Abstract

We have observed that treatments with salicylic acid (SA) or gentisic acid (GA) induced resistance to RNA pathogens such as ToMV and CEVd in tomato and *Gynura auriantiaca*, respectively. Accumulation of SA and GA has been found to occur in plants infected by these pathogens, thus pointing out a possible defence role of both molecules. To study the molecular basis of the observed induced resistance to RNA pathogens the induction of silencing-related genes by SA and GA was considered. For that purpose, we searched for tomato genes which were orthologous to those described in *Arabidopsis thaliana*, such as *AtDCL1, AtDCL2*, *AtDCL4*, *AtRDR1*, *AtRDR2* and *AtRDR6*, and we tracked their induction in tomato along virus and viroid infections. We observed that CEVd significantly induced all these genes in tomato, with the exception of *ToRDR6*, being the induction of *ToDCL4* the most outstanding. Regarding the ToMV asymptomatic infection, with the exception of *ToRDR2*, we observed a significant induction of all the indicated silencing-related genes, being *ToDCL2* the most induced gene. Subsequently, we analyzed their transcriptional activation by SA and at the time when ToMV was inoculated on plants. *ToDCL2*, *ToRDR1* and *ToRDR2* were significantly induced by both SA and GA, whereas *ToDCL1* was only induced by SA. Such an induction resulted more effective by SA treatment, which is in agreement with the stronger SA-induced resistance observed. Our results suggest that the observed delay in the RNA pathogen accumulation could be due to the pre-induction of RNA silencing-related genes by SA or GA.

Highlights

- Treatments with salicylic acid or gentisic acid induce resistance to RNA pathogens
- Induction of tomato silencing-related genes by RNA pathogen infections is studied
- Induction of RNA silencing by SA or GA treatments is analysed in tomato plants
- New connexions between RNA silencing and the SA or GA response are proposed

Key words

Resistance, salicylic acid, gentisic acid, gene silencing, ToMV, CEVd, tomato

1. Introduction

Higher plants can not move, and are consequently exposed to a wide array of damaging agents, including biotic (viroids, viruses, bacteria, fungi, insects) and abiotic (drought, salinity, heat, cold, soil toxicity) environmental aggressions. To cope with these continuous challenges, plants have evolved broad and efficient mechanisms to obtain an adequate defence. One prominent defensive response of plants against pathogen attack is the synthesis of a remarkably vast array of low molecular weight compounds with disparate functions in plant-pathogen interactions (Dixon, 2001).

Resistance in plants can also be induced by treatments with a variety of abiotic and biotic elicitors that lead to the activation of plant defence responses and consequently enhanced protection against disease. The interest in developing agents for activating the plant's own resistance mechanisms to control diseases has increased over the last years (Walters et al., 2005).

The simple phenolic salicylic acid (SA) plays a very important role in plant defence, and is crucial to establish the resistance response in many plant-pathogen interactions (Delaney et al., 1994). In this sense, elevated levels of SA are found in many incompatible plant-pathogen interactions but also in some compatible ones (Baebler et al., 2011; Bellés et al., 1999) and exogenous treatments with SA induce in the plant the synthesis of a group of proteins that are collectively referred to as pathogenesis-related (PR) proteins, many of which have antimicrobial properties (van Loon et al., 2006). Moreover, *nahG* plants, which are unable to accumulate SA, are more susceptible to pathogens (Vlot et al., 2009). Several studies have demonstrated that exogenous treatments with SA induce resistance to different pathogens in tomato (Mandal et al., 2009; Meher et al., 2011; Shang et al., 2011; Spletzer and Enyedi, 1999) and other plant species (Edgar et al., 2006; Saikia et al., 2003; Wang and Liu, 2012).

Gentisic acid (GA), a metabolic derivative of SA, has been proposed as a signal molecule for plant defence response in compatible, non-necrotizing, interactions (Bellés et al., 1999). Interestingly, GA has been found to accumulate in different compatible plant-pathogen interactions (Bellés et al., 2006; Bellés et al., 2008), being produced at much higher levels than other signal molecules such as SA. Moreover, exogenous GA

elicits the induction of a specific set of pathogenesis-related proteins (PRs) which are not induced by SA (Bellés et al., 1999; Bellés et al., 2006; Lisón et al., 2013) Additionally, NMR-based metabolomics showed that glycosylated gentisic acid was the most important induced metabolite of viroid-infected plants (López-Gresa et al., 2010). Unlike SA, which is conjugated to glucose, GA is conjugated to xylose by a specific GA-xylosyl-transferase in tomato (Fayos et al., 2006; Tárraga et al., 2010). Salicylic acid has been widely related to the establishment of plant resistance against pathogen attack. In contrast, little is known on the possible role of gentisic acid in plant patogen resistance.

Gene silencing is a kind of induced resistance which occurs in plants infected with RNA pathogens. It consists of a series of interconnected pathways that limit the synthesis, stability and translatability of foreign or aberrant RNAs. These processes have three stages in common: (a) presence or formation of double-stranded RNA (dsRNA), (b) processing of this dsRNA into small RNA fragments called sRNA, and (c) incorporation of the sRNA into a complex that is associated specifically with the complementary RNA target. As a result, the negative regulation of RNA target occurs, producing the so-called gene silencing (Brodersen and Voinnet, 2006; Carr et al., 2010).

Different genes have been described to participate in the gene silencing mechanisms. In the first stage (a), the double-stranded RNA may be already present or may result from the copy of a single-stranded RNA (ssRNA) into a dsRNA by an RNA-dependent RNA polymerase (*RDR* or *RdRP*). In the second stage (b), the dsRNA is processed into sRNAs by Dicer-like endoribonucleases (*DCL*). Finally (c), the sRNA generated by DCLs are incorporated into the *AGO-RISC* complex, where they will serve as a template to direct the specific degradation of the RNA target (Brodersen and Voinnet, 2006).

RNA silencing could explain different induced-resistance phenomena such as cross-protection between homologous viruses. In this case, an infection using a mild virus strain protects the plant against a severe strain of the same virus or a closely related one, a mechanism that has remained unexplained for more than 75 years (Ziebell and Carr, 2010). Similarly, RNA silencing has also been described for viroids, which are single-stranded, circular, non-coding RNAs that infect plants, causing devastating diseases (Flores et al., 2005). It has been observed that the infection by members of the two viroid families Pospiviroidae and Avsunviroidae is followed by the accumulations of their corresponding sRNAs (Navarro et al., 2012; Sano et al., 2010).

Several lines of evidence indicate a possible overlap between RNA-silencing pathways and signal transduction pathways governed by SA. Among them, NtRdRP activity has been found to increase in tobacco plants following SA treatment (Xie et al., 2001). Orthologous genes of *NtRDR1* have been also characterized in other species, such as *Nicotiana glutinosa* (*NgRDR1*), *N. benthamiana* (*NbRDR1m*), *A. thaliana* (*AtRDR1*), *Medicago truncatula* (*MtRDR1*) and rice (*OsRDR1*), being all of them induced by both viral infection and SA treatments (Liu et al., 2009; Quilis et al., 2008; Yang et al., 2004; Yu et al., 2003). Besides, certain viral silencing suppressor proteins also suppress SA-mediated defence (Ji and Ding, 2001). Environmental conditions (e.g., temperature) influence the induction of SA-dependent defence responses and RNA silencing (Szittya et al., 2003; Wang et al., 2009). Therefore, it should be considered that there are links between RNA silencing and SA-mediated defence, although the way these connections are established remains unclear (Carr et al., 2010).

Using different plant-pathogen systems we have investigated the involvement of salicylic acid and gentisic acid in the induction of resistance to RNA pathogens.

Symptom development and pathogen spread was monitored, and the expression of different silencing-related genes was also analysed along the infections and treatments. Our results indicate that salicylic acid and gentisic acid might play a role in plant resistance against RNA pathogens.

2. Methods

2.1. Plant material and growth conditions

Seeds from tomato (*Solanum lycopersicum* cv. "Rutgers") were surface-sterilized with bleach before use. Plants were grown in 15-cm diameter pots containing a mixture of peat and vermiculite mixed 1:1 (one plant per pot), and were sub-irrigated with Hoagland solution. Plants were cultivated in a greenhouse at a temperature of 25/30º C (day/night) with a relative humidity between 50 to 70 % and a photoperiod of 16 h light/8 h darkness. Four-week-old tomato plants were used in all the experiments described in this article.

Gynura aurantiaca DC. plants were grown as previously described (Bellés et al., 1990) from rooted cuttings in a greenhouse at 30/24º C (day/night), with a relative humidity between 50 to 70% and a photoperiod of 16 h light/8 h darkness.

2.2. Treatments with SA and GA

Tomato plant treatments were carried out by stem-feeding, according to the method described by Gu *et al.* (Gu et al., 2000). For that purpose, four-week-old Rutgers tomato plants were excised with a scalpel just above the cotyledons, and the stems were immersed in 50 ml plastic tubes containing either buffer 50 mM sodium phosphate pH 7.4 (control plants), 1 mM SA in phosphate buffer or 2 mM GA, prepared in the same buffer. These concentrations were chosen according to our previous experience since GA conjugates to a much greater extent than SA after the corresponding treatments (Tárraga et al., 2010). Treatments were performed in a growth chamber at a constant temperature of 24º C and a photoperiod of 16 h light (55-75 μmol/m^2/s) and 8 h darkness. Both third and fourth leaves from the explants were harvested at the indicated times and immediately frozen in liquid nitrogen for RTqPCR analysis. For virus inoculation, explants were transferred to water 48 h after the corresponding treatment, and the third leaf was then inoculated with ToMV.

Treatments of *Gynura aurantiaca* plants were performed by spraying plants until run-off with 1 mM SA or 2 mM gentisic acid (sodium salt forms) supplemented with 0.05% Tween 20 (Sigma) as a wetting agent. Equivalent control plants were only sprayed with 0.05% Tween 20. Treatments were repeated three times a week during the entire experiment.

2.3. Pathogen inoculations

Infection of Rutgers tomato plants with the Murakishi PV-0143 strain of Tomato Mosaic Virus (obtained from Leibniz Institute DSMZ, Braunschweig, Germany) was performed with a viral extract obtained from leaves of ToMV-infected tomato plants that were homogenized in 10 mM sodium phosphate buffer (pH 7.2), 0.5% sodium bisulphite, 0.5% diethyldithiocarbamic acid (1 g leaf material in 20 mL buffer) as

described (Bellés *et al.,* 2006). Tomato explants were placed in 50-ml plastic tubes containing distilled water, and the third leaf (numbered from cotyledons to apex) was dusted with carborundum (particle size 0.037 mm). One millilitre of viral extract or buffer (mock-inoculated control) was applied to the dusted leaf by gently rubbing the adaxial surface with a soft camel-hair brush. The third and fourth leaves (local and distal tissues, respectively) were harvested separately at the indicated times, and immediately frozen in liquid nitrogen.

Inoculation of tomato and *Gynura aurantiaca* plants with citrus exocortis viroid (CEVd) was performed following the protocol described by Bellés *et al.* (Bellés et al., 1990).

2.4. Protein extraction and electrophoretic analysis

Leaf tissue was harvested under liquid nitrogen and stored at −80º C until use. Protein extracts of tomato leaves were performed by homogenization in extraction buffer (50 mM Tris-HCl, pH 7.5, 15 mM 2-mercaptoethanol), as described in (Rodrigo et al., 1993). Proteins were separated by SDS-PAGE and stained with Coomassie Brilliant Blue R-250 as described by Conejero and Semancik (Conejero and Semancik, 1977).

2.5. RNA extraction and treatment

Total RNA of tomato leaf tissue was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. RNA was further precipitated by adding one volume of LiCl 6 M, and then the pellet was washed with LiCl 3 M and dissolved in RNase-free water. Finally, 2 units of TURBO DNase (Ambion) were added per µL RNA to remove contaminating genomic DNA.

2.6. Quantitative RT-PCR analysis

One µg total RNA was used to obtain the corresponding cDNA target sequences using an oligo$(dT)_{18}$ primer and the PrimeScript RT reagent kit (Perfect Real Time, Takara) according to the manufacturer's protocol. PCR was carried out in the presence of the double-stranded DNA-specific dye Power SYBR Green PCR Master Mix (Applied Biosystems). Amplification was monitored in real time with the 7500 FAST Real-Time PCR System (Life Technologies). The PCR primers used for RT-PCRs are shown in Table S2.

2.7. Statistical analysis

The parameter used to perform the statistical analysis was the plant “Infectivity Index”. It consists of the total number of days that each plant presents symptoms, thus providing a measure of the delay in the onset of symptoms. Data from a total of 122 plants corresponding to 3 independent experiments were used to perform a Kruskal-Wallis test (non-parametric test equivalent to the one-way ANOVA).

For the qRT-PCR analysis shown in Figures 3 and 4, a *t*-test analysis was performed. Comparisons between multiple groups (SA-treated, GA-treated and buffer-treated plants) in Figure 5 were made by analysis of variance (ANOVA) for each time point. A p value < 0.05 was considered significant.

The SPSS v.19 package (IBM) was used for all the statistical analysis.

3. Results

3.1. SA and GA treatments induce resistance to ToMV in tomato

The tomato-ToMV plant-pathogen interaction has been used to study the effect of SA and GA treatments on the plant resistance to RNA pathogens. Along the infection, the accumulation of SA and GA has been described to occur (Bellés et al., 1999). To study the effect of these compounds on resistance to the virus, tomato plants were cut and stem-fed either with SA, GA or buffer solutions for 48 hours. Explants were then transferred to water and inoculated with ToMV as described in Methods. Samples of both the inoculated leaves (local tissues) and the immediately superior leaves (distal tissues) were taken at different time points. As a marker of the infection, we analyzed the accumulation of the ToMV coat protein by SDS-PAGE.

As Figure 1A shows, all the inoculated (local) tissues accumulated viral coat protein at the studied times. However, plants treated with SA seemed to have slightly lower levels of viral protein when compared with GA-treated or control plants.

Regarding the distal samples (Figure 1B), the coat protein appeared to be detectable upon eight days after inoculation. At 9 dpi, a systemic infection was clearly noticeable in the buffer-treated samples, whilst the plants treated with GA and SA presented significantly lower levels of the viral protein, therefore indicating a systemic resistance of tomato plants to ToMV induced by GA and SA. Finally, a slight difference can be observed after 10 dpi between the GA-induced resistance and the resistance mediated by SA, the last one being the most effective.

These results indicate that SA or GA treatments induce systemic resistance to ToMV in tomato plants.

3.2. SA and GA treatments induce a delay in the appearance of CEVd symptoms in Gynura aurantiaca

It has been reported that *Gynura aurantiaca* plants infected with *Citrus exocortis viroid* (CEVd) accumulate GA and SA (Bellés et al., 2006). To study whether the SA- or GA-induced resistance also took place in this systemic RNA-based infection, *Gynura aurantiaca* plants were sprayed either with these compounds or buffer solutions, and then inoculated with CEVd. Treatments were repeated throughout the experiment, as described in Methods.

A follow-up of the onset of symptoms was carried out by observing the appearance of leaf epinasty and reduced growth during the development of the infection. As shown in Figure 2, plants treated with GA or SA presented a delay in the onset of symptoms with respect to control plants treated with buffer. Similarly to what was found for the viral infection in tomato, the resistance induced by SA was more effective than that promoted by GA.

To check the statistical significance of the differences shown in Figure 2, we used the "Infectivity Index" of the plant as a measure of the delay in the onset of symptoms. This parameter consists of the total number of days that each plant presents symptoms, in such a way that the smaller this number is, the higher the delay would be in the appearance of symptoms. Infectivity indexes from the plants corresponding to 3 independent experiments were used to perform a statistical analysis (see Methods). We observed statistically significant differences (p-value = 0.018) among the infectivity indexes for Control, GA and SA treated plants (Supplementary Data Table S1A).

Comparing the groups in pairs (Control-GA and Control-SA), the differences were statistically significant (p-value = 0.046 and p-value = 0.008, respectively), thus indicating that both treatments provoke a significant delay in the onset of symptoms with respect to the control plants treated with buffer. There was not significant difference between the infectivity index of the GA and SA treated plants (p-value = 0.403) plants (Supplementary Data Table S1B). Therefore, these results indicate that both SA and GA treatments induce resistance to CEVd in plants of *Gynura aurantiaca* in a similar way.

3.3. Tomato genes involved in gene silencing are induced by ToMV and CEVd

To study the molecular basis of the observed resistance to RNA pathogens, we considered to test the induction by SA or GA of gene silencing related genes, in tomato. So we decided to look for the tomato DCL, AGO and RDR genes and study their pattern of induction by virus and viroid infections. Based on the corresponding sequences of *Arabidopsis thaliana* and using the Blast tool of Solgenomics database (http://solgenomics.net/tools/blast/index.pl) we found the tomato orthologues of the *AtDCL1, AtDCL2*, *AtDCL4*, *AtRDR1*, *AtRDR2* and *AtRDR6* genes (Supplementary Data Table S2). To test the correct assignment of the tomato sequences with the RDRs and the DCLs described in *Arabidopsis thaliana*, we performed a phylogenetic tree from sequence alignments for each group of genes (See Supplementary Data, Figure S1). The different RDRs and DCLs proposed for tomato matched precisely to those described in Arabidopsis. Moreover, the tomato sequence orthologous to the Arabidopsis RDR1 turned out to be the so-called RdRp, described by Schiebel and collaborators in 1998. All these data confirmed the alignment-based correspondences.

To verify the possible involvement of these candidate sequences in tomato RNA gene silencing, we tested their expression upon CEVd and ToMV infection. For virus infection, real-time-quantitative PCR was performed for tomato *RDR* and *DCL* sequences, using RNAs from ToMV-infected tomato plants taken 7 days after the inoculation, as well as their corresponding uninfected controls (see Methods). We observed a statistically significant induction of all the silencing-related genes as a consequence of the asymptomatic virus infection, with the exception of the RNA-dependent RNA polymerase *ToRDR2*, which displayed only a minor increase in its expression which resulted to be non-significant (Figure 3). ToMV infection provoked the strongest induction on *ToDCL2*, reaching levels up to 40 times higher than those found in control plants.

To track the expression pattern of these genes for a RNA pathogen of a different nature, tomato seedlings were inoculated with citrus exocortis viroid (CEVd). A systemic infection was clearly established twenty days after the inoculation, whose symptoms consisted of leaf epinasty and reduced growth. At that stage, we carried out RT-qPCR analyses for the indicated silencing-related genes from CEVd-infected tomato plants and their corresponding controls. Figure 4 shows that CEVd infection significantly induced all these genes, with the exception of the *ToRDR6* polymerase, whose expression was barely higher and statistically non-significant as compared to control plants. Unlike what happened in viral infection, the induction of *ToDCL4* appeared to be the most prominent in viroid-infected plants.

3.4. Tomato genes involved in gene silencing are induced by GA and SA treatments

In an attempt to explain the increased resistance to virus found in tomato plants treated with SA and GA, we considered to study the effect of these signal molecules in the expression of tomato *DCLs* and *RDRs*. For that purpose, we performed the tomato stem feeding treatments, either with SA, GA or buffer, collecting tissue samples at different time points: 0, 4, 8, 12, 24 and 48 hours of incubation. These samples were analyzed by RT-qPCR with specific primers of the tomato silencing related genes. We used the induction of tomato Pathogenesis-Related PR1 and P23 genes as positive control of the treatments with SA and GA, respectively (Figure 5A) (Bellés et al., 1999).

As Figure 5B shows, SA treatment significantly induced the expression of both *ToDCL1* and *ToDCL2* endonucleases, showing a maximum at 12 h and 8 h, respectively. Treatment with GA treatment resulted in a statistically significant induction of *ToDCL2,* with a maximum at 4 hours. Therefore, the induction of *ToDCL2* by GA seemed to be faster than the effect caused by SA*,* although less intense. A slight induction of the three tomato DCLs was observed in the buffer-treated plants, which might be due to the wound produced by the stem feeding treatment. On the other hand, a strong and significant repression of *ToDCL4* was detected upon treatments with SA or GA.

As far as the tomato *RDR* polymerases are concerned (Figure 5C), *ToRDR1* and *ToRDR2* were found to be strongly induced by SA, displaying a maximum at 8 and 12 hours of treatment, respectively. Interestingly treatment with SA produced a 100-fold increase in the expression of *ToRDR1* (also known as RdRP). On the other hand, we could detect a significant induction of *ToRDR1* and *ToRDR2* by GA after 4 hours of treatment. Similarly to what was observed for *ToDCL4*, no induction of *ToRDR6* was detected upon SA or GA treatments. Again, we observed a slight induction of the three RDRs in the control plants, which could be due to the wound caused by the treatment. Similarly to what was found for *ToDCL4*, a repression of *ToRDR6* was observed in the plants treated with SA or GA.

These results indicate that the observed GA or SA induced resistance could be related with the induction of the genes involved in the gene silencing response in tomato. The greater efficiency of the SA-induced resistance correlates with the stronger SA induction of the *DCLs* and *RDRs,* with respect to the effect produced by GA.

4. Discussion

An increased resistance to ToMV has been found in tomato plants treated with SA or GA. Besides, *Gynura aurantiaca* plants sprayed with SA or GA also present a delay in the onset of symptoms of CEVd infection. Both pathogens are RNA-based, and the accumulation of SA and GA has been described to occur in both plant-pathogen systemic interactions (Bellés et al., 1999).

It is well known that SA plays a very important role in plant defence signalling (Vlot et al., 2009). Moreover, a number of studies have demonstrated that exogenous treatments with SA induce resistance to different pathogens in different species (Edgar et al., 2006; Mandal et al., 2009; Meher et al., 2011; Saikia et al., 2003; Shang et al., 2011; Spletzer and Enyedi, 1999; Wang and Liu, 2012). Studies with different plant-pathogen systems establishing a compatible, non-necrotizing interaction, have reported a significant accumulation of gentisic acid (GA) in infected plants (Bellés et al., 1999; Bellés et al., 2006; López-Gresa et al., 2010). Furthermore, exogenous applications of this compound

induce defensive responses different than those mediated by salicylic acid (SA). Therefore, gentisic acid has emerged as a signalling molecule additional to salicylic acid in compatible infections (Bellés et al., 1999). In this paper, we have demonstrated that treatments with GA can also induce resistance to RNA pathogens, in a similar way as its metabolic precursor SA.

It is commonly admitted that the SA-induced resistance is due in part to the induction of PR proteins (Bowles, 1990; Hammerschmidt, 2009; Van Loon and Van Strien, 1999). Many PRs have antibacterial or antifungal activity (Van Loon and Van Strien, 1999), however none of the described PR proteins seems to have antiviral activity (Cutt et al., 1989; Linthorst et al., 1989). Related to this, it has been described that the induced tomato resistance to different RNA viruses caused by treatments with jasmonic acid and SA, seems not to be related with PR proteins (Shang et al., 2011). Therefore, it is of great interest to explore the molecular basis of the observed SA and GA induced plant resistance by considering the induction of genes not related with defence against herbivores, fungi or bacteria, but specifically targeted to pathogens based on RNA, such as viruses and viroids.

To this respect, RNA silencing is a powerful defence mechanism against RNA pathogens, with a great specificity and adaptability (Ding and Voinnet, 2007). Moreover, there are several lines of evidence indicating that there is some kind of cross-talk between defences mediated by SA and the RNA silencing in various species, such as tobacco, *Arabidopsis thaliana* or *N. benthamiana* (Carr et al., 2010; Xie et al., 2001; Yang et al., 2004; Yu et al., 2003). Therefore we decided to study the involvement of SA and GA in the regulation of the silencing-related genes implicated in the observed resistance to RNA pathogens.

A search in the Sol Genomics Network database allowed us to obtain the tomato orthologous sequences of *AtDCL1, AtDCL2, AtDCL4, AtRDR1, AtRDR2* and *AtRDR6* which perfectly matched to the genes that have been very recently described (Bai et al., 2012). The expression of DCL endonucleases has been reported to be activated as a result of virus or viroid infections (Carr et al., 2010). On the other hand, the RDRs have also been implicated in viral and viroidal silencing. Therefore, we tested whether the expression of the tomato RDR and DCL genes that we found was altered as a result of a viral or viroidal infection.

In ToMV-infected tomato plants, we have observed an induction of all the analyzed genes with the exception of *ToRDR2*, being *ToDCL2* the most prominently induced gene.

In *Arabidopsis thaliana*, genetic studies have revealed the hierarchical access of DCL4 and DCL2 to viral dsRNA. DCL4 is the primary sensor of viral dsRNAs and produces 21 nt vsiRNAs, the most abundant size class in infected tissues. DCL2 acts as a DCL4 surrogate to generate 22 nt vsiRNAs (Deleris et al., 2006; Llave, 2010). However, we have observed that *ToDCL2* is induced by ToMV in a greater extend that *ToDCL4*. In accordance to our results, a significant induction of the *DCL2* gene has been reported in tomato upon *Pepino mosaic virus* (PepMV) infection, being the induction of *DCL4* by PepMV much less pronounced (Hanssen et al., 2011). The authors claim that such an induction of *DCL2* could be indicative of the presence of a virus-encoded silencing suppressor that could be interfering with DCL4 activity. Finally, although DCL1 is a minor contributor to vsiRNA formation in plants infected with RNA viruses (Llave, 2010), DCL1 is thought to excise hairpin-like structures from primary transcripts in dsDNA-Cauliflower mosaic virus-infected plants, thereby facilitating access by the

other DCLs (Moissiard and Voinnet, 2006). In accordance to this, we have also observed a slight induction of DCL1 in tomato by ToMV.

On the other hand, numerous studies indicate that the RDRs are involved in antiviral silencing, since plants that present alterations in the activity of these proteins show an increased susceptibility to RNA and DNA viruses. Although they may act in a complementary and coordinated manner, it seems that the RDRs display specific sensitivities to different viruses (Llave, 2010). This could explain the fact that *ToRDR1* and tomato *ToRDR6* were induced by ToMV, while *ToRDR2* was not altered. The induction of *RDR1* by virus has been described in other plant-virus systems such as *Arabidopsis thaliana* infected with the crucifer-infecting Tobacco Mosaic Virus (TMV-cg) (Yu et al., 2003). Similar results have been reported in tobacco plants infected either with Tobacco Mosaic Virus (TMV) (Xie et al., 2001), with Plum Pox Virus (Alamillo et al., 2006) or with Tomato Ring Spot Virus (Jovel et al., 2011). Accordingly, to what has been described in tomato plants infected with PepMV (Hanssen et al., 2011), we have observed a slight up-regulation of *ToRDR6* in tomato plants infected by ToMV.

Concerning the viroid infection, we have observed an induction of all the analyzed genes in tomato, with the exception of *ToRDR6*, being the induction of *ToDCL4* the most outstanding.

The endonuclease DCL1 has been implicated in the cleavage of RNA molecules derived from DNA viruses and also in the formation of microRNAs (Brodersen and Voinnet, 2006). Since viroids are RNA molecules, the observed DCL1 induction could be related to the accumulation of microRNAs acting on plant mRNAs. Therefore, this induction is in accordance with the hypothesis, supported by many authors, that the viroid-activated silencing would be part of the viroid disease itself more than a plant defence mechanism (Gómez et al., 2008; Markarian et al., 2004; Martínez et al., 2010; Matousek et al., 2007; Navarro et al., 2012; Papaefthimiou et al., 2001; Wang et al., 2004). To our best knowledge, the induction patterns of DCL2 and DCL4 by viroid infection have not been studied so far, although their activities on viroid RNA particles are supposed to occurred since the accumulation of VdsRNAs have been described several times (Navarro et al., 2009).

The *ToRDR1* viroid induction has also been described in tomato plants infected by Potato Spindle Viroid (Schiebel et al., 1998). Although we have observed no induction *ToRDR6* by viroid, *RDR6* has been involved in the induction of viroid symptoms in plants of *Nicotiana benthamiana* infected with Hop Stunt Viroid (HSVd) (Gómez et al., 2008) or by the Potato Spindle Tuber Viroid (PSTVd) (Di Serio et al., 2010). Finally, some authors have proposed that *RDR2,* which we have observed to be induced by CEVd in tomato, could be implicated in the recognition of (+) viroid RNAs (Navarro et al., 2009).

Our results on the induction pattern of the tomato orthologous silencing-related sequences upon viral and viroid infection confirmed the possible involvement of these genes in RNA silencing mechanisms. Consequently, we analyzed the transcriptional induction of the tomato silencing-related genes by SA and GA, in order to explain the previously observed induced resistance.

As mentioned above, there are different lines of evidence that correlate the SA-induced response and RNA silencing (Carr et al., 2010). Among these, it has been described that RDR1 is induced as a consequence of SA treatments in different plant species (Liu et al., 2009; Quilis et al., 2008; Xie et al., 2001; Yang et al., 2004; Yu et al., 2003). Our results are added to those already described, since they indicate that *ToRDR1* is induced

as a result of exogenous SA treatments in tomato. Similarly, *ToDCL1*, *ToDCL2* and *ToRDR2* are also induced by SA treatments, which further reinforce the relationship between SA-induced response and RNA silencing.

Exogenous GA treatments were also found to induce RNA silencing-related genes in tomato, such as *ToRDR1* and *DCL2*, although their effect appeared to be weaker. The involvement of gentisic acid in gene silencing could explain the increased gene expression and activity of RDR1 in systemic infections caused by a compatible strain of TMV (Xie et al., 2001), although the accumulation of SA does not occur in these plants (Malamy et al., 1990).

In addition, our results seem to indicate that wounding could be activating some tomato DCLs and RDRs. Accordingly, evidences that correlate wound response and gene silencing have been reported. For instance, RdR1 has been found to mediate the resistance of *Nicotiana attenuata* to herbivore attack in nature (Pandey and Baldwin, 2007), and DCL2 and DCL4 are proposed to participate in the anti-herbivore defences (Bozorov et al., 2012). On the other hand, we have observed a repression of *ToDCL4* and *ToRDR6* upon treatment with SA and GA, when compared with buffer-treated plants. This could be due to an antagonistic effect of the SA or GA treatments on the wounding response. The antagonism between SA and jasmonic acid, which is the signal molecule implicated in the wound response, has been well established in the literature (reviewed in Thaler et al., 2012 and Derksen et al., 2013).

In summary, we have observed that both SA and GA treatments induce resistance to different RNA pathogens and activate RNA silencing-related genes in tomato. Treatments involving stronger induction of these genes correlated with the stronger resistance. Besides, we have found that shorter GA and SA treatments, which are insufficient to produce an effective accumulation of PR proteins (Matsuoka and Ohashi, 1986) produce a delay in virus accumulation (see Supplementary Figure S2). Therefore, our results suggest that the observed induced resistance could be due to the induction of RNA silencing mechanisms, thus reinforcing the connections between gene silencing and plant pathogen defence.

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Contributions

The work presented here was carried out in collaboration between all authors. Purificación Lisón, Ismael Rodrigo, José María Bellés and Vicente Conejero defined the research theme. Laura Campos, Pablo Granell, Susana Tárraga and Pilar López-Gresa designed methods and experiments, carried out the laboratory experiments, analyzed the

data and interpreted the results. Purificación Lisón and Ismael Rodrigo wrote the paper. All authors have contributed to, seen and approved the manuscript.

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Figure legends.

Figure 1. SDS-PAGE analysis of soluble proteins from Tomato Mosaic Virus (ToMV)-infected tomato leaves. Prior inoculation, plants were pre-treated either with buffer (B), gentisic acid (GA) or salicylic acid (SA), and leaf tissue samples were collected at the indicated days post-inoculation (dpi). Protein size markers (kDa) are indicated on the left. Arrow on the right indicates the ToMV coat protein (CP). **A)** Protein profile of infected (local) leaves. **B)** Protein profile of systemic (distal) leaves.

Figure 1

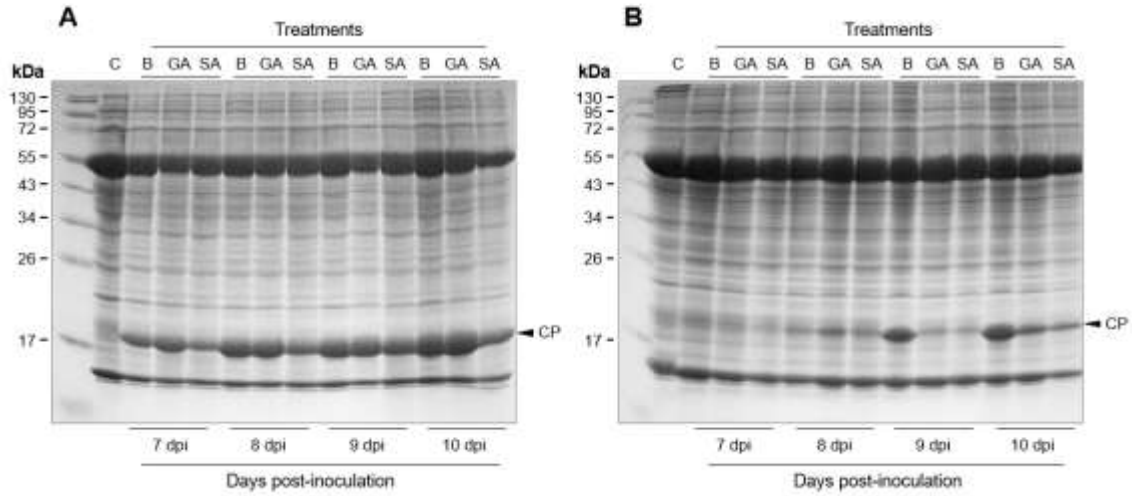


Figure 2. Disease development in *Gynura aurantiaca* plants infected with CEVd. Plants were treated either with buffer, gentisic acid (GA) or salicylic acid (SA). **A)** Comparison of CEVd symptoms (stunting, leaf epinasty and rugosity) in *Gynura aurantiaca* plants treated with buffer, GA or SA. **B)** Evolution of the number of *Gynura aurantiaca* plants showing CEVd symptoms at the indicated days post-inoculation.

Figure 2

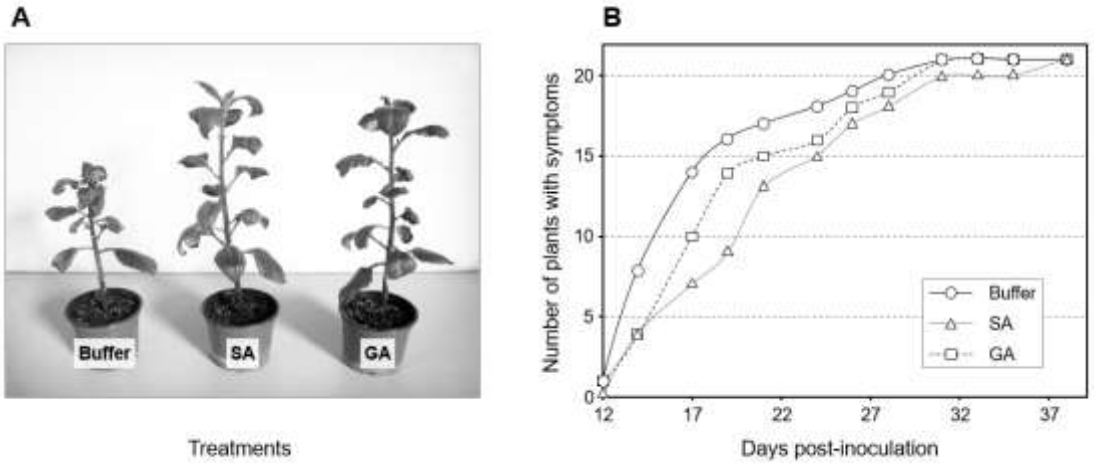


Figure 3. Expression levels of tomato *DCL1*, *DCL2*, *DCL4*, *RDR1*, *RDR2* and *RDR6* genes in ToMV-infected tomato plants determined by real-time qRT-PCR analysis. Values were first normalized to the actin expression level and then made relative to the mRNA amount in the control, which refers to non-infected plants. Three biological repetitions were carried out. Expression levels are expressed as means +/- standard errors. A *t*-test analysis was performed. Asterisks (*) indicate statistical significance with p value < 0.05. Double asterisks (**) indicate statistical significance with p value < 0.01.

Figure 3

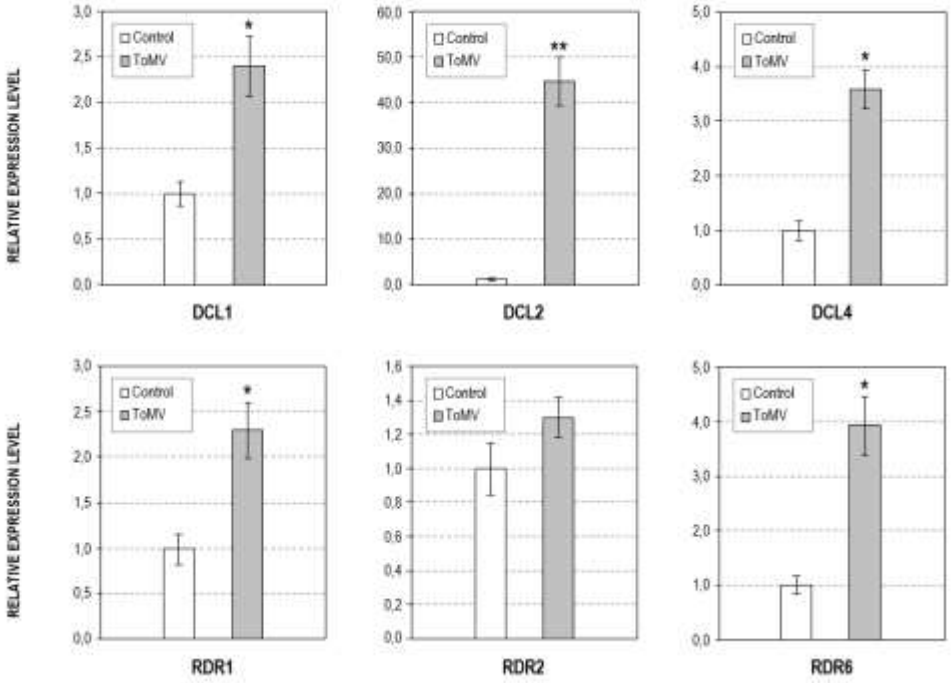


Figure 4. Expression levels of tomato *DCL1*, *DCL2*, *DCL4*, *RDR1*, *RDR2* and *RDR6* genes in CEVd-infected tomato plants determined by real-time qRT-PCR analysis. Values were first normalized to the actin expression level and then made relative to the mRNA amount in the control, which refers to non-infected plants. Three biological repetitions were carried out. Expression levels are expressed as means +/- standard errors. A *t*-test analysis was performed. Asterisks (*) indicate statistical significance with p value < 0.05. Double asterisks (**) indicate statistical significance with p value < 0.01.

Figure 4

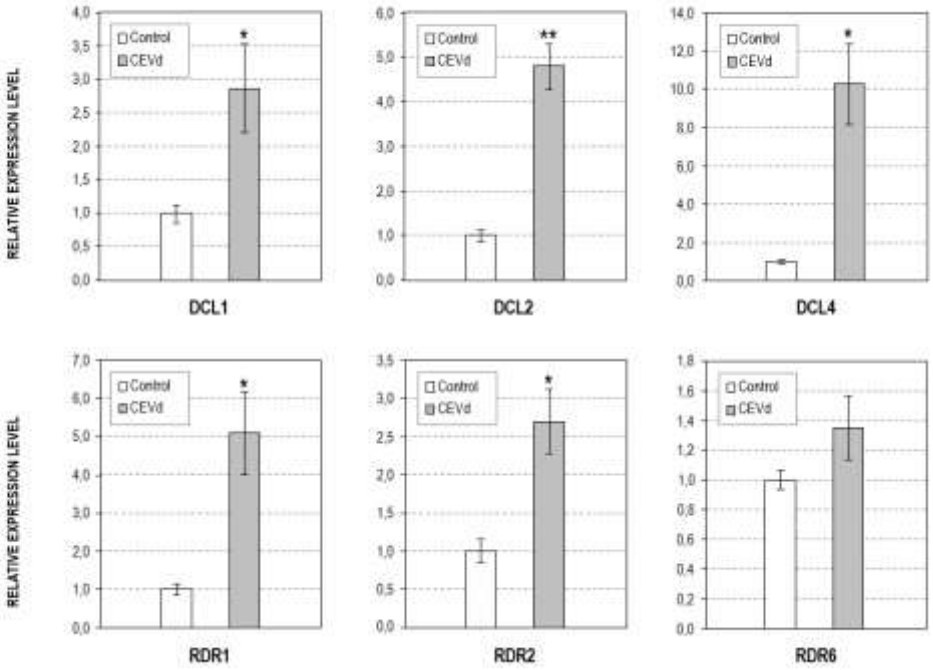
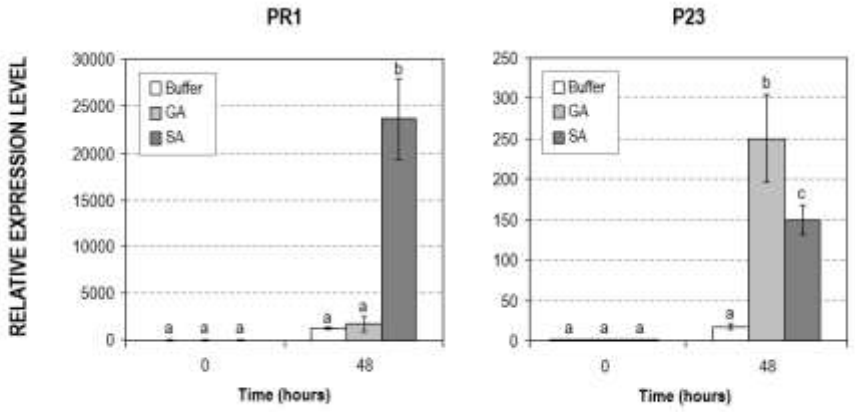


Figure 5. Expression levels of silencing-related genes in tomato plants treated either with buffer, gentisic acid (GA) or salicylic acid (SA). The mRNA levels of *DCL1*, *DCL2* and *DCL4* **(5A)**; *RDR1*, *RDR2* and *RDR6* **(5B)** and the pathogenesis-related proteins PR1 and P23 **(5C)** were determined by real-time qRT-PCR analysis. Values were first normalized to the actin expression level and then made relative to the mRNA amount in the control, which refers to non-infected plants. Three biological repetitions were carried out. Expression levels are expressed as means +/- standard errors. Comparisons between multiple groups (SA-, GA- and buffer-treated plants) were performed by analysis of variance (ANOVA) for each time point. The same letter indicates that there are no significant differences (p value < 0.05).

Figure 5A



Supplementary Data

Table S1

A

Group	N	Average ranks	Chi-squared	Degrees of freedom	*p*-value
Control	42	73.12	7.847	2	0.018
GA	42	58.36			
SA	38	52.13			

B

Groups in pairs	*p*-value
Control - GA	0.046
Control - SA	0.008
GA - SA	0.403

Table S1. Kruskal-Wallis test for infectivity indexes of CEVd-infected *Gynura aurantiaca* plants treated with either buffer, gentisic acid (GA) or salicylic acid (SA). **A)** Statistical parameters of the Kruskal-Wallis test for the infectivity indexes of Control, GA-treated or SA-treated *Gynura aurantiaca* plants infected with CEVd. N = number of plants. **B)** *p*-values of the Kruskal-Wallis tests for the infectivity indexes when groups were compared in pairs.