Activation of senescence-associated *Dark-inducible (DIN)* genes during infection contributes to enhanced susceptibility to plant viruses

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SUMMARY

Virus infections in plants cause changes in host gene expression that are common to other environmental stresses. In this work, we found extensive overlap in the transcriptional responses between *Arabidopsis thaliana* plants infected with *Tobacco rattle virus* (TRV) and plants undergoing senescence. This is exemplified by the up-regulation during infection of several senescence-associated *Dark-inducible (DIN)* genes, including *AtDIN1* (*Senescence 1*, *SEN1*), *AtDIN6* (*Asparagine synthetase 1*, *AtASN1*) and *AtDIN11*. *DIN1*, *DIN6* and *DIN11* homologues were also activated in *Nicotiana benthamiana* in response to TRV and *Potato virus X* (PVX) infection. Reduced TRV levels in RNA interference (RNAi) lines targeting *AtDIN11* indicate that *DIN11* is an important modulator of susceptibility to TRV in *Arabidopsis*. Furthermore, low accumulation of TRV in *Arabidopsis* protoplasts from RNAi lines suggests that *AtDIN11* supports virus multiplication in this species. The effect of *DIN6* on virus accumulation was negligible in *Arabidopsis*, perhaps as a result of gene or functional redundancy. However, TRV-induced silencing of *NbASN*, the *DIN6* homologue in *N. benthamiana*, compromises TRV and PVX accumulation in systemically infected leaves. Interestingly, *NbASN* inactivation correlates with the appearance of morphological defects in infected leaves. We found that *DIN6* and *DIN11* regulate virus multiplication in a step prior to the activation of plant defence responses. We hypothesize on the possible roles of *DIN6* and *DIN11* during virus infection.

Keywords: *Arabidopsis thaliana*, *Dark-inducible (DIN)* genes, *Nicotiana benthamiana*, plant viruses, *Potato virus X*, senescence-associated genes, *Tobacco rattle virus*.

INTRODUCTION

Plants respond to pathogen infection by reprogramming host gene expression, which includes, among others, the induction of

a suite of defence- and pathogenesis-related genes (van Loon *et al.*, 2006; Wise *et al.*, 2007). The expression of defence-related genes is also triggered during senescence, a process of programmed cell death that occurs in response to developmental, physical, physiological and hormonal cues (Buchanan-Wollaston *et al.*, 2003; Gepstein *et al.*, 2003; Lim and Nam, 2005; Quirino *et al.*, 2000). Interestingly, senescence-associated genes are activated during pathogen attack, which is suggestive of common events or signalling pathways in the control of gene expression in these two overlapping processes (Lin and Wu, 2004; Quirino *et al.*, 1999). Some senescence-enhanced genes are detected during the hypersensitive response against incompatible bacteria and fungi and against virulent or avirulent pathogens that cause necrosis (Pontier *et al.*, 1999; Schenk *et al.*, 2005). This observation could lead to the misleading assumption that cell death is the necessary point of convergence between defence and senescence. However, senescence-related genes are also expressed at elevated levels during compatible interactions between plants and viruses (Espinoza *et al.*, 2007; Whitham *et al.*, 2003). Hence, the execution of the cell death programme seems to be uncoupled from the initial regulation of senescence. Cellular stress is the most likely factor explaining why senescence-related genes are activated during pathogen-activated responses. Indeed, many stress-inducible genes are up-regulated during senescence, which implies that cells undergoing senescence are subjected to stress conditions (Gepstein *et al.*, 2003; Weaver *et al.*, 1998). In addition, stresses caused by drought, darkness, sugar starvation, wounding or leaf detachment initiate the senescence programme (Lin and Wu, 2004; Weaver *et al.*, 1998). The extent and significance of this overlap and the precise roles of pathogen- and senescence-responsive pathways remain largely unknown.

The production of reactive oxygen species (ROS) is a unifying response to multiple stresses and, consequently, has been proposed as a common signal controlling gene expression in stressed plants (Love *et al.*, 2005; Quirino *et al.*, 2000; Torres and Dangl, 2005). Senescence and pathogen infection are both accompanied by a certain degree of cellular damage caused by oxidative stress and, accordingly, ROS detoxification genes are stimulated in both types of process (Buchanan-Wollaston *et al.*, 2003; Espinoza *et al.*, 2007; Schippers *et al.*, 2008; Whitham

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et al., 2003). For instance, glutamate dehydrogenase (GDH), which is activated by both senescence and viral or bacterial infections, is highly expressed under oxidative stress (Pageau *et al.*, 2006). Chemical signal compounds, such as salicylic acid (SA) and methyl jasmonate (MeJA), which have defensive and senescence-promoting functions, regulate the expression of certain senescence- and pathogen-associated genes (Schenk *et al.*, 2005). Changes in the source-to-sink balance, normally associated with pathogen invasions, are also pivotal in regulating leaf senescence (Herbers *et al.*, 2000; Masclaux *et al.*, 2000; Shalitin and Wolf, 2000).

In this study, we have identified a set of genes, collectively named *Dark-inducible (DIN)*, that actively respond to *Tobacco rattle virus* (TRV) infection in *Arabidopsis thaliana*. *DIN* genes are induced in dark-adapted and senescing leaves (Fujiki *et al.*, 2001, 2005; Lin and Wu, 2004; Quirino *et al.*, 2000), and inspection of *Arabidopsis* microarray data using Genevestigator indicates that *DIN* genes also respond to biotic stimuli. This finding suggests that *DIN* genes could participate in cellular events that are common to both senescence and pathogen invasion (Zimmermann *et al.*, 2004). However, although some *DIN* genes, such as *DIN1*, *DIN6* and *DIN11*, have been used previously as markers to characterize senescence-associated responses, their regulation and function during compatible interactions between viruses and host plants are largely unknown (Lam *et al.*, 1994; Oh *et al.*, 1996; Schenk *et al.*, 2005). Here, we show that *DIN6*- and *DIN11*-encoding enzymatic activities may play critical roles in the modulation of plant susceptibility to virus infection.

RESULTS

Gene expression profiling reveals significant similarities between TRV infection and senescence in *Arabidopsis*

To discriminate the group of genes whose expression was similarly regulated in response to TRV infection and senescence, we compared microarray data of the *Arabidopsis* response to TRV with gene expression profiling data during developmental or dark-induced leaf senescence (Buchanan-Wollaston *et al.*, 2005; Fernandez-Calvino *et al.*, 2014; Lin and Wu, 2004). Global transcriptomic changes in TRV-infected *Arabidopsis* plants were documented at 8 days after inoculation (dai), when TRV has been shown to exhibit the highest peak of genomic RNA accumulation (Fernandez-Calvino *et al.*, 2014). TRV-responsive genes (Bonferroni-corrected $P < 0.01$) were compared with the list of genes described above. We found a group of 68 genes that responded similarly to virus infection, dark-induced senescence and age-mediated (natural) senescence [Table S1 (Supporting Information) and Fig. 1A]. Interestingly, 61% of the TRV-responsive genes (724 genes) were altered in the same direction

(either induced or repressed) when senescence was artificially induced by darkness or carbon starvation, and 8% (97 genes) were shared between TRV infection and developmental leaf senescence. In both cases, the number of common genes in the intersection between TRV infection and dark-induced senescence or developmental senescence was higher than that expected in a random distribution ($P < 0.0001$), indicating significant commonalities in their transcriptional responses (Fig. 1A). Using an independent set of plants, and consistent with microarray data, quantitative reverse transcription-polymerase chain reaction (qRT-PCR) revealed increased transcript levels of the stress-inducible *Senescence-associated gene 13* (*AtSAG13*, *At2g29350*) at 8 dai (Fig. 1B) (Gan and Amasino, 1997; Weaver *et al.*, 1998).

Despite the extensive degree of overlap in the transcriptomic responses of TRV-infected leaves and leaves undergoing senescence (Fig. 1A), TRV infection in *Arabidopsis* apparently proceeds in the absence of macroscopic alterations that could be diagnostic of accelerated induction of leaf senescence (Lin *et al.*, 2009; Quirino *et al.*, 2000). To accurately determine the effect of virus infection on plant growth and development, we measured a series of morphological traits in vegetative and reproductive organs in mock-inoculated and TRV-infected plants at 16 and 29 dai. None of the growth-related traits tested differed significantly between healthy and TRV-infected plants (Figs 1C and S1, see Supporting Information). Likewise, equivalent numbers of viable seeds and seed weight were produced by infected and mock-inoculated plants (Fig. 1C). Chlorophyll breakdown during chloroplast disassembly is a typical visible manifestation of senescence (Quirino *et al.*, 2000). However, chlorophyll content remained at comparable levels in mock-inoculated and TRV-infected leaves at 16 and 29 dai (Figs 1D and S1). We also determined electrolyte leakage to assess whether virus infection caused membrane damage. Our results indicated a similar percentage of electrolyte leakage in leaves inoculated with TRV and in upper non-inoculated TRV-infected leaves with respect to the corresponding non-infected leaves, even at later time points after infection (Figs 1E and S1). Collectively, the overlap in the transcriptional responses between TRV infection and both types of senescence suggests a significant interaction between these two stress-related processes, which, however, did not reflect a premature ongoing senescence-like phenotype.

TRV stimulates the expression of *DIN* genes in *Arabidopsis*

Among the genes that exhibited similar responsiveness to TRV in different *Arabidopsis* organs (F. J. del Toro and C. Llave, unpublished data), we identified several senescence-associated genes catalogued as *DIN* genes. The gene expression data and predicted functions of *Arabidopsis* *DIN* genes deduced from their amino acid sequences are shown in Table 1. To validate microarray-based

Fig. 1 Analysis of senescence-associated traits during *Tobacco etch virus* (TRV) infection in Arabidopsis. (A) Venn diagram to illustrate the number of specific and common misregulated genes in dark-induced senescence, natural senescence and TRV infection in Arabidopsis. Genes showing significant TRV responsiveness were compared with Affymetrix expression data from the two types of senescence: 1, Lin and Wu (2004); 2, Buchanan-Wollaston *et al.* (2005). The numbers of genes within the intersections are higher than those expected by chance. To test whether the significance of matches was higher than explained by random sampling, we assumed a Poisson distribution from a mean value calculated as $\mu = G1 \times (G2/Gt)$, as any gene in G1 has a G2/Gt possibility of belonging to G2, where G1 and G2 are the subsets of genes to be compared and Gt is the total number of genes represented in the microarray. (B) Time-course expression of the stress senescence marker *AtSAG13* in TRV-infected plants examined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The means of three measurements $\pm$ standard deviation (SD) are shown. Values are relative to those in mock-inoculated plants at 3 days after inoculation (dai) which were arbitrarily assigned a value of unity after normalization to the *AtCBP20* internal control. Asterisks indicate significant difference from the mock-inoculated control ($P < 0.001$, Duncan's multiple range test). (C) Effect of TRV infection on the number of rosette and cauline leaves, secondary stems, inflorescences and siliques, and stem length (cm) at 16 dai. Total seed number and weight (g) were used to quantify progeny production. (D) Total chlorophyll content in Arabidopsis leaves at 16 dai. (E) Activation of cell death measured by leakage of ions in mock-inoculated and TRV-infected plants at 16 dai. The means and SD of three independent replicates are shown.

expression of *DIN* genes in TRV-infected Arabidopsis, we conducted qRT-PCR on the same pooled RNA extracts as used for microarray hybridization, as well as on an independent set of samples (not used for microarrays), to confirm reproducibility in their expression patterns. According to this analysis, *AtDIN1* (also *Senescence 1*, *AtSEN1*) and *Asparagine synthetase 1* (*AtASN1*), the Arabidopsis homologue of *DIN6* (herein referred to as *DIN6* for simplicity), transcripts were significantly more abundant in

TRV-infected tissue than in the non-infected control in the two sets of samples collected from leaves, inflorescences and roots (Fig. 2). These results corroborated the microarray-based prediction that *AtDIN1* and *AtDIN6* were strongly and widely activated by TRV. qRT-PCR demonstrated that *AtDIN11*, which was not represented in the CATMA (Complete Arabidopsis Transcriptome MicroArray) used, was induced by TRV in all three tissues analysed using independent RNA preparations (Fig. 2). In this study, we focus on

Table 1 Microarray-based expression changes in *Dark-inducible (DIN)* genes in three Arabidopsis organs.

Gene	AGI	Protein description	Fold change†		
			Leaves	Inflorescences	Roots
<i>DIN1</i>	At4g35770	Senescence-associated gene 1 (SEN1)	7.31***	4.75***	3.58***
<i>DIN2</i>	At3g60140	β-Glucosidase 30, Senescence-associated gene 2 (SEN2)	1.04	−0.98	1.04
<i>DIN3</i>	At3g06850	Dihydrolipoamide branched chain acyltransferase (BCE2)	1.06	1.44*	1.86***
<i>DIN4</i>	At3g13450	Branched chain α-keto acid dehydrogenase E1 β	1.91**	2.65***	1.5**
<i>DIN6</i>	At3g47340	Glutamine-dependent asparagine synthetase 1 (ASN1)	2.05***	3.01***	1.89***
<i>DIN9</i>	At1g67070	Phosphomannose isomerase 2 (PMI2)	−0.96	−0.97	1.00
<i>DIN10</i>	At5g20250	Raffinose synthase 6 (RS6)	−0.82	4.5***	1.94***
<i>DIN11</i>	At3g49620	2-Oxoacid-dependent dioxygenase	ND‡	ND	ND

†Bold numbers indicate significant differences in gene expression between mock-inoculated and *Tobacco rattle virus* (TRV)-infected plants. Differences from control values were significant at $P < 1 \times 10^{-12}$ (***), $P < 0.01$ (**) or $P < 0.05$ (*) (Bonferroni-corrected method).

‡ND, not detected.

Fig. 2 Expression of *Dark-inducible (DIN)* genes in response to *Tobacco rattle virus* (TRV) infection in Arabidopsis. Transcript levels of *AtDIN1* (*Senescence 1, SEN1*), *AtDIN6* (*Asparagine synthetase 1, ASN1*) and *AtDIN11* were examined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) in two independent experiments (Exp #1 and Exp #2). The means of three measurements $\pm$ standard deviation (SD) are shown. Values are given relative to those in mock-inoculated plants that were arbitrarily assigned a value of unity after *AtTUB5* normalization. Samples were collected from roots and non-inoculated rosette leaves at 8 days after inoculation (dai) and inflorescences at 12 dai. Differences from control values were significant at $P < 0.001$ (*) or $P < 0.05$ (†) (Duncan's multiple range test).

AtDIN1, *AtDIN6* and *AtDIN11*, given that these three genes were significantly up-regulated and exhibited broad responsiveness throughout the plant (Table 1).

Microarray data indicated the deregulation of other dark-responding genes in the presence of TRV, although changes in

their expression patterns were not very reproducible between organs or batches of plants. In general, *AtDIN3* and *AtDIN10* were activated by TRV in both inflorescences and roots, and their mRNA accumulation patterns were confirmed by qRT-PCR in independent RNA samples (Fig. S2, see Supporting Information). Although

microarray data did not reflect differential changes in *AtDIN3* or *AtDIN10* expression in leaves, qRT-PCR showed that *AtDIN10* mRNA levels were substantially reduced using independent samples from infected relative to non-infected leaves, whereas *AtDIN3* was either repressed or unaffected by TRV (Fig. S2). We found that *AtDIN2* transcripts, which did not exhibit TRV responsiveness on the basis of microarray-based expression profiling, were significantly more abundant in both sets of samples from roots and leaves when assessed using qRT-PCR (Fig. S2). Finally, our gene expression tests indicated that *AtDIN9* was consistently up-regulated in inflorescences on TRV challenge, whereas transcript accumulation varied when roots and leaves were analysed (Fig. S2).

AtDIN11 enhances susceptibility to TRV in Arabidopsis

To assess the contribution of *DIN1*, *DIN6* and *DIN11* to TRV infection, we used Arabidopsis knockout lines of these genes to monitor virus accumulation. qRT-PCR revealed that TRV accumulated in single *din1* or *din6* mutants to the same levels as in the wild-type background, suggesting that neither *AtDIN1* nor *AtDIN6* was critical for viral susceptibility in Arabidopsis (Fig. 3A,B). To test *AtDIN11* contribution to TRV infection, we obtained transgenic Arabidopsis plants harbouring a hairpin dsRNA-based RNA interference (RNAi) construct targeted against the *AtDIN11* gene. Total RNA from systemically infected rosette leaves of two independent homozygous T3 Arabidopsis lines (L10 and L21) infected with TRV

was subjected to qRT-PCR experiments. In these plants, *AtDIN11* transcript levels were reduced by more than 80% compared with wild-type controls, demonstrating the effective silencing of the targeted gene (Fig. 3C). Silencing of *AtDIN11* in both L10 and L21 RNAi lines correlated with a significant two-fold reduction in TRV accumulation compared with wild-type plants at 5 and 8 dai (Fig. 3C). Interestingly, TRV levels showed large differences between independent replicates and genetic backgrounds at 16 dai (data not shown), suggesting that the influence of *DIN11* on TRV accumulation could be restricted to the early time points of infection during which virus proliferation is particularly dynamic. To test this hypothesis, we isolated protoplasts from Arabidopsis rosette leaves and subjected them to transfection with the infectious pTRV1 vector. qRT-PCR of total RNA extracted at 24 h post-transfection demonstrated that viral replication was severely compromised in both L10 and L21 lines (Fig. 3D). This result was highly reproducible, confirming that a functional *DIN11* was required to allow virus multiplication. No major visible morphological differences were observed between knockout or RNAi lines and wild-type plants (data not shown).

TRV and *Potato virus X* (PVX) promote the expression of *NbNRIP1*, *NbASN* and *NbDIN11* in infected *Nicotiana benthamiana*

We were interested in determining whether TRV-mediated induction of *DIN1*, *DIN6* or *DIN11* was unique to Arabidopsis or whether

Fig. 3 Effect of *Dark-inducible* (*DIN*) genes on *Tobacco rattle virus* (TRV) susceptibility in Arabidopsis. Transcript accumulation, determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR), is given relative to that in wild-type plants that were assigned a value of unity after normalization. The absolute quantification of TRV RNA1 is given as the number of viral copies per nanogram of total RNA. Means of three measurements $\pm$ standard deviation (SD) are shown. Asterisks indicate statistical significance versus the wild-type control ($P < 0.001$, Duncan's multiple range test). (A, B) Analysis of *AtDIN1* (A) and *AtDIN6* (B) expression and accumulation of TRV RNA1 in TRV-infected Arabidopsis leaves of *din1* (A) and *din6* (B) mutants at 8 days after inoculation (dai). (C) Analysis of *AtDIN11* expression and accumulation of TRV genomic RNA1 in infected leaves of Arabidopsis *DIN11* RNAi lines (L10 and L21) at 5 dai (absolute quantification) and 8 dai (relative quantitation). (D) Accumulation of TRV RNA1 in Arabidopsis protoplasts from L10 and L21 lines 24 h after transfection.

Fig. 4 Expression of *Dark-inducible* (*DIN*) homologues in *Nicotiana benthamiana* in response to *Tobacco rattle virus* (TRV) and *Potato virus X* (PVX). Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of NbNRIP1 (*N receptor-interacting protein 1*), NbASN (*Asparagine synthetase 1*) and NbDIN11 expression in upper non-inoculated leaves of plants infected with TRV-GFP or PVX-GFP. Accumulation levels are referenced to the mock-inoculated controls that were arbitrarily assigned a value of unity after normalization to the NbACT internal control. The means of three measurements $\pm$ standard deviation (SD) are shown. Asterisks indicate statistical significance versus the mock-inoculated control ($P < 0.001$, Duncan's multiple range test). GFP, green fluorescent protein.

these genes responded to TRV infection in other susceptible hosts. To test this possibility, we first conducted a BLAST search using Arabidopsis *DIN* genes as queries to find putative *DIN* homologue genes in the *N. benthamiana* expressed sequence tag (EST) database. Our homology-based search identified chloroplastic *N receptor-interacting protein 1* (NbNRIP1) (EU332891) and NbASN (GQ354808) as genes homologous to Arabidopsis AtDIN1 (At4g35770) and AtDIN6 (At3g47340), respectively. We failed to identify sequences with extensive sequence similarity to Arabidopsis AtDIN11 (At3g49620) through database mining in *N. benthamiana* (*N. benthamiana* transcriptome v5 primary transcripts database; http://sydney.edu.au/science/molecular_bioscience/benthamiana/, University of Sydney, Sydney, Australia). However, we were able to amplify an *N. benthamiana* cDNA product using PCR primers designed from highly conserved sequence domains of *Solanum tuberosum* (DQ200393.1) and *S. lycopersicum* ESTs (AK321665.1), that each showed a sequence identity of approximately 67% at the nucleotide level with AtDIN11, suggesting that a *DIN11* homologue exists in the *N. benthamiana* genome.

We then inoculated *N. benthamiana* plants with TRV, and mRNA accumulation was registered for each *DIN* gene in the upper non-inoculated leaves. qRT-PCR assays indicated that NbNRIP1, NbASN and putative NbDIN11 transcripts accumulated to significantly higher levels in *N. benthamiana* in the presence of TRV relative to mock-inoculated plants (Fig. 4). These results were fully reproducible in independent experiments and demonstrated that the *DIN* homologues NbNRIP1, NbASN and NbDIN11 are induced by TRV in different host species.

We next wanted to test whether these three *DIN* genes were activated in *N. benthamiana* in response to other RNA viruses. To test this, we agroinjected *N. benthamiana* leaves with a PVX-green fluorescent protein (PVX-GFP) clone, and the upper non-inoculated leaves were analysed for transcript accumulation. qRT-PCR revealed that NbNRIP1, NbASN and NbDIN11 endogenous transcripts were increased significantly by more than 50% in PVX-infected plants relative to mock plants agroinfiltrated with an empty vector (Fig. 4). These results demonstrate that *DIN1*, *DIN6* and *DIN11* are potential targets of regulation during infections by different viruses in different hosts.

Suppression of NbASN compromises susceptibility to TRV and PVX in *N. benthamiana*

To investigate the influence of NbNRIP1, NbASN and NbDIN11 genes on TRV susceptibility in *N. benthamiana*, we tested whether the inactivation of the endogenous *DIN* genes affected the multiplication and spread of TRV in this species. We took advantage of our TRV infectious clone as a vector for virus-induced gene silencing (VIGS) to knock down the expression of the target *DIN* genes. A mixture of *Agrobacterium* cultures containing TRV1 and a recombinant TRV2 clone that carried a fragment of the target gene was co-infiltrated on *N. benthamiana* leaves as described previously (Fig. 5A) (Liu *et al.*, 2002). Expression of the targeted *DIN* transcripts was monitored alongside TRV accumulation using qRT-PCR in the upper non-infiltrated leaves 17 days after agroinjection. A TRV-GFP clone that carried the GFP coding sequence gene was used as a control. To accurately amplify endogenous NbNRIP1, NbASN and NbDIN11 transcripts, qRT-PCR primers were designed from regions located outside of those cloned into the corresponding TRV derivatives used for silencing.

When a recombinant TRV-NbNRIP1 infectious clone that harboured a fragment of the NbNRIP1 gene was introduced into *N. benthamiana* plants, we observed a five-fold decrease in endogenous NbNRIP1 transcript levels in the upper leaves relative to plants infected with a TRV-GFP construct (Fig. 5B). Similarly, infection of *N. benthamiana* plants with the recombinant TRV-NbDIN11 clone that carried a fragment of the potato homologue of *DIN11* resulted in a two- to five-fold reduction in the relative NbDIN11 transcript levels compared with control plants (Fig. 5C and data not shown). These results demonstrate the robustness of silencing of the target genes and validate our experimental approach. qRT-PCR also revealed that TRV-genomic RNA accumulated in these silenced leaves to the same high levels as those in plants infected with TRV-GFP, suggesting that silencing of NbNRIP1 or NbDIN11 was not sufficient to alter virus accumulation (Fig. 5B,C).

Agrobacterium-mediated infiltration of TRV-NbASN, which contained a fragment of the *DIN6* Arabidopsis homologue, resulted in approximately 90% inhibition of NbASN expression in systemi-

Fig. 5 Effect of silencing of *Dark-inducible (DIN)* genes on virus accumulation and symptom expression in *Nicotiana benthamiana* plants. Expression values, determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR), are relative to those in plants infected with *Tobacco rattle virus*-green fluorescent protein (TRV-GFP) or *Potato virus X*-green fluorescent protein (PVX-GFP) that were assigned a value of unity after NbACT normalization. The means of three measurements $\pm$ standard deviation (SD) are shown. Asterisks indicate significant difference from the TRV-GFP/PVX-GFP control ($P < 0.001$, Duncan's multiple range test). (A) Genome organization of the TRV-based virus-induced gene silencing (VIGS) vectors used for silencing of endogenous *N. benthamiana* *DIN* homologues. (B, C) Analysis of *NbNRIP1* (B) and *NbDIN11* (C) expression and TRV RNA1 accumulation in upper leaves of plants infected with TRV-NbNRIP1 (B) or TRV-NbDIN11 (C). (D) Analysis of *NbASN* (*Asparagine synthetase 1*) expression and accumulation of TRV RNA1 in upper leaves infected with TRV-NbASN. Symptoms associated with silencing of *NbASN* in TRV-infected leaves are shown relative to the asymptomatic infection caused by TRV-GFP. (E) Analysis of *NbASN* expression and PVX genomic RNA accumulation in upper leaves infected with PVX-NbASN and PVX-GFP. The genome organization of the PVX derivative used to silence *NbASN* is shown.

cally infected leaves relative to plants infected with the TRV-GFP control (Fig. 5D). Strikingly, suppression of *NbASN* expression in plants infected with TRV-NbASN was reproducibly accompanied by the appearance of dispersed chlorotic areas and leaf deformation that became conspicuous in the youngest apical leaves at approximately 15 dai (Fig. 5D). In addition, *NbASN*-silenced plants were far less susceptible to TRV as they accumulated 60%–90% less viral genomic RNA1 than non-silenced plants agroinoculated

with the control TRV-GFP construct (Fig. 5D). Reduction of TRV levels occurred only in *NbASN*-silenced plants, but not in *NbNRIP1*- or *NbDIN11*-silenced plants, which supports the conclusion that low TRV accumulation is caused by knockdown of *NbASN*.

Given that *NbASN* was inducible by PVX in *N. benthamiana* (Fig. 4), we wondered whether silencing of *NbASN* could also interfere with PVX accumulation in this species. To explore this

idea, we adopted a PVX-based silencing strategy to challenge *N. benthamiana* plants with an infectious PVX construct containing a fragment of NbASN. qRT-PCR analysis showed that the endogenous NbASN transcript levels were diminished by about 35% in systemically infected leaves relative to those observed in plants infected with a PVX-GFP vector (Fig. 5E). Remarkably, partial silencing of NbASN transcripts was sufficient to cause a significant reduction (80%) in PVX RNA levels in the upper infected leaves (Fig. 5E). In these plants, upper silenced leaves infected with PVX-NbASN did not exhibit a distinguishable phenotype with respect to the non-silenced leaves infected with PVX-GFP (data not shown), perhaps because suppression of the endogenous NbASN gene was incomplete in PVX-NbASN-infected plants.

Reduced susceptibility to TRV in *AtDIN11*- or NbASN-silenced plants is not caused by a general activation of defence genes

Our findings that virus accumulation was drastically impaired in Arabidopsis plants in which *DIN11* transcripts were silenced or in *N. benthamiana* leaves with reduced NbASN transcript levels prompted us to investigate whether the inactivation of *DIN11* or NbASN stimulated an antiviral plant defence response. To test this possibility, we first conducted expression analyses of *Pathogenesis-related 1* (*AtPR1*, At2g14610) and *Plant defensin 1.2* (*AtPDF1.2*, At5g44420) genes in the hairpin *DIN11* RNAi transgenic L10 and L21 lines. *PR1* and *PDF1.2* are commonly used to monitor SA- and jasmonic acid (JA)-dependent defence responses (Loake and Grant, 2007). The SA pathway is typically activated during senescence and virus infections, and resistance to certain RNA viruses is SA dependent (Mayers *et al.*, 2005; Whitham *et al.*, 2006). Application of MeJA is known to inhibit the replication of several viruses in compatible interactions (Jameson and Clarke, 2002). Interestingly, we found that *AtPDF1.2* expression was compromised in both *AtDIN11*-silenced RNAi lines compared with Col-0, whereas *AtPR1* expression somehow differed between the independent RNAi lines tested (Fig. S3, see Supporting Information). qRT-PCR using RNA preparations from TRV-infected plants revealed that *AtPR1* and *AtPDF1.2* transcripts accumulated to comparable levels to those found in non-infected plants. This finding suggests that lower TRV levels in our *DIN11* RNAi lines were not caused by the elevated expression of these two defence markers (Fig. 6A).

To determine whether silencing of NbASN in *N. benthamiana* plants infected with TRV-NbASN correlated with the activation of defensive genes, the expression of NbPR1 (G1FNL9) and *Heat-shock protein 70* (NbHSP70, GQ354819.1) was examined using the same RNA samples as employed for NbASN and TRV accumulation in transient expression assays. We chose to test NbHSP70 expression because HSPs are associated with plant virus replica-

tion (Aranda *et al.*, 1996; Mine *et al.*, 2012). qRT-PCR revealed that NbPR1 and NbHSP70 transcript levels were reduced by nearly 70%–80% in NbASN-silenced leaves infected with TRV-NbASN compared with non-silenced, TRV-GFP-infected controls (Fig. 6B).

We next investigated the expression pattern of NbPR1 and NbHSP70 in TRV-infected leaves in which a 35S-driven construct containing the Arabidopsis *AtDIN6* homologue gene had been agroinjected 2 days prior to virus inoculation. Transient accumulation of *AtDIN6* transcripts was corroborated by qRT-PCR compared with control plants infiltrated with an empty vector (Fig. 6C). We found that TRV levels in leaves expressing *AtDIN6* were similar to those observed in control leaves infiltrated with an empty vector (Fig. 6C). Both NbPR1 and NbHSP70 mRNAs accumulated in the presence of *AtDIN6* to the same levels as those observed in control plants infiltrated with empty vector (Fig. 6C). These results suggest that TRV accumulation and induction of NbPR1 and NbHSP70 are unaffected by the ectopic expression of *AtDIN6* (Fig. 6C).

DISCUSSION

In our study, we have shown that TRV induces host gene responses that are similar to those activated during senescence, even though TRV infection occurs in the absence of morphological manifestations of disease. This means that the activation of senescence genes during TRV infection does not necessarily imply the initiation of the senescence syndrome. Collectively, *DIN* genes are activated in leaves undergoing senescence, as well as in leaves subjected to stimuli that normally lead to senescence, such as darkness, phosphate or carbon starvation, wounding or treatment with photosynthesis inhibitors (Fujiki *et al.*, 2001). Microarray-based analysis of gene expression indicates that the up-regulation of *DIN* genes also occurs during responses induced by compatible and incompatible fungal and bacterial pathogens, or on treatment with defence-inducing chemical signals (Genevestigator data) (Schenk *et al.*, 2005). Therefore, it seems reasonable to anticipate that *DIN* genes are part of the basal metabolic responses used by cells to cope with stresses that may be common to senescence and pathogen infections. However, like many other stress-inducible genes, the function of *DIN* genes remains elusive at this time. In addition, how these genes contribute to the infectious process is an important question that remains unresolved. The main objective of this study was to examine the effect of *DIN1*, *DIN6* and *DIN11* on plant susceptibility to virus infection. We found that *AtDIN1*, *AtDIN6* and *AtDIN11* were broadly regulated in Arabidopsis in response to TRV, whereas transcripts from NbNRIP1, NbASN and NbDIN11 accumulated to significantly increased levels in *N. benthamiana* leaves infected with TRV or PVX. This finding indicates that *DIN* induction probably represents a common response to different viruses in different compatible host species.

Fig. 6 Effect of *AtDIN11* (*Dark-inducible 11*) and *NbASN* (*Asparagine synthetase 1*) on the expression of defence-related genes in *Tobacco rattle virus* (TRV)-infected plants. Expression values, determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR), are the means of three measurements $\pm$ standard deviation (SD). Asterisks indicate statistical significance versus the controls ($P < 0.001$, Duncan's multiple range test). (A) Expression analysis of *AtPR1* (*Pathogenesis-related 1*) and *AtPDF1.2* (*Plant defensin 1.2*) in upper non-inoculated rosette leaves of *Arabidopsis* *DIN11* RNA interference (RNAi) lines (L10 and L21) systemically infected with TRV. Values in infected Col-0 plants were assigned a value of unity after *AtCBP20* normalization. (B) Expression analysis of *NbPR1* and *NbHSP70* (*Heat-shock protein 70*) in upper *N. benthamiana* leaves systemically infected with TRV-*NbASN* relative to control plants infected with TRV-GFP which were assigned a value of unity after *NbACT* normalization. (C) Expression analysis of *NbPR1* and *NbHSP70* in TRV-infected *N. benthamiana* leaves that transiently overexpressed the *Arabidopsis AtDIN6* homologue relative to the control leaves in which an empty vector (EV) was agroinoculated (this sample was assigned a value of unity after *NbACT* normalization).

Our data indicated that *DIN1* was apparently irrelevant for virus infection in both *Arabidopsis* and *N. benthamiana*. Similarly, *AtDIN1* was shown to be dispensable for disease resistance or tolerance to bacterial and fungal pathogens, although *AtDIN1* is strongly elicited during defence responses against these pathogens (Oh *et al.*, 1996; Schenk *et al.*, 2005). However, *NbNRIP1*, the *N. benthamiana* homologue of *AtDIN1*, is required for an effective N-mediated defence response to *Tobacco mosaic virus* (TMV) in *N. benthamiana* (Caplan *et al.*, 2008). VIGS of *NbNRIP1* partially abolishes the function of N and allows TMV to spread systemically (Caplan *et al.*, 2008). *NbNRIP1* mediates the association of the N immune receptor and the TMV-encoded 50-kDa helicase (p50) effector protein to assemble a core recognition complex that allows the plant to detect TMV in infected cells (Caplan *et al.*, 2008). Although TRV accumulation was not altered in *NbNRIP1*-silenced plants, it would be interesting to investigate whether *NbNRIP1* could also mediate the recognition of putative TRV effector proteins by host immune receptors. The 5'-upstream sequence of the *DIN1* gene contains a TCA motif (TCATCTTCTT) that is highly conserved among genes induced by

various stresses (Goldsbrough *et al.*, 1993; Oh *et al.*, 1996). Therefore, this sequence motif may be involved in the induction of *DIN1* homologues under the stress caused by TRV or PVX in infected cells.

We found that *AtDIN6* was not crucial for TRV infection in *Arabidopsis*. Likewise, *AtDIN6* was unnecessary for basal defence and resistance against bacterial and oomycete infection in *Arabidopsis* (Hwang *et al.*, 2011). However, inactivation of the *AtDIN6* homologue *NbASN* affected negatively TRV and PVX replication in *N. benthamiana*. Interestingly, despite the important role of *NbASN* in viral infection in this species, ectopic expression of *AtDIN6* had no significant effects on TRV accumulation. A possible explanation is that *DIN6/ASN*-related functions are not affected by elevated expression of *DIN6/ASN*-coding genes, as opposed to what is expected from its genetic inactivation. Yet, additional experiments are needed to assess the functionality of the *Arabidopsis* homologue *AtDIN6* in *N. benthamiana*. We demonstrated that VIGS of *NbASN* was accompanied by a dramatic reduction in TRV or PVX genomic RNA relative to that in non-silenced control plants. It is worth noting that TRV-mediated

silencing of the target NbASN transcripts occurs concomitantly with severe morphological defects in the most apical leaves infected with TRV. In contrast, PVX-NbASN-infected plants, in which silencing of NbASN was only partial, developed normally with an absence of morphological abnormalities. These observations raise the question of whether symptoms are uniquely caused by disruption of NbASN functions or are also influenced by specific host–virus interactions. Interestingly, VIGS of the *DIN6* homologue gene of *Capsicum annuum* (*CaAS1*) was found to interfere with pathogen susceptibility by enhancing bacterial infections in pepper (Hwang *et al.*, 2011). The apparent discrepancies between our observations and those reported by Hwang *et al.* (2011) could be a result of the inherent differences in the experimental approaches and pathogens tested, with different strategies used to infect the plants.

Our results demonstrated that *AtDIN11* influences virus susceptibility in Arabidopsis, as manifested by reduced TRV levels in upper non-inoculated leaves of *DIN11* RNAi lines at early stages of infection. Furthermore, our results suggested that *AtDIN11* supports TRV replication, as the accumulation of viral genomic RNA was partially inhibited in Arabidopsis protoplasts isolated from knockout lines compared with wild-type Col-0. The reduced susceptibility observed in *AtDIN11*- and NbASN-silenced plants cannot be attributed to a boost in the induction of general defence responses during infection, as none of the defence markers tested in this study showed a changed in expression profile in response to viral infection. This observation is remarkable and supports the idea that *AtDIN11* and NbASN have a direct impact on viral susceptibility by modulating virus accumulation upstream and independent of the activation of defence genes. Nevertheless, we cannot exclude the possibility that other components of the defensive reaction respond positively to *AtDIN11* or NbASN levels to dampen virus accumulation. Why TRV and PVX are unable to replicate efficiently when *AtDIN11* or NbASN activity is compromised is unknown.

DIN6 encodes a glutamine-dependent asparagine synthetase that regulates the levels of asparagine in the dark or under carbon-limiting conditions (Lam *et al.*, 1994, 1995, 1998). Induction of *DIN6* in virus-infected plants is presumably linked to the deaminating activity of GDH, a component of senescence and plant defence responses that is up-regulated in Arabidopsis infected with TRV (Fernandez-Calvino *et al.*, 2014; Pageau *et al.*, 2006). Glutamate deamination by GDH releases significant amounts of free ammonium that must be rapidly eliminated to avoid deteriorating effects within the cell (Lam *et al.*, 1998; Masclaux-Daubresse *et al.*, 2006). Given that asparagine is viewed as an ammonia detoxification product, it is sensible to propose that the activation of *DIN6* could contribute to counteract the toxic effects of excess ammonium produced during the infection and to help maintain cell integrity, thus allowing effective virus multiplication. Gene or functional redundancy could

explain, at least in part, why the genetic inactivation of *AtDIN6* in the Arabidopsis *din6* mutant has no influence on TRV susceptibility. Indeed, the Arabidopsis genome contains two additional asparagine synthetase homologous genes (*AtASN2* and *AtASN3*), which encode a novel class of asparagine synthetase enzyme with enhanced ammonia-dependent activity (Lam *et al.*, 1998). *DIN11* encodes a protein similar to plant dioxygenases that shows a requirement for 2-oxoglutarate as a co-substrate (Fujiki *et al.*, 2001; Prescott and John, 1996). Therefore, like *DIN6*, the activation of *DIN11* in TRV-infected plants could be coupled to the GDH-catalysed deamination of glutamate to 2-oxoglutarate (Masclaux-Daubresse *et al.*, 2006). However, the precise function of *DIN11* remains largely unknown.

To date, very limited information is available concerning the role of host factors in compatible plant–virus interactions. Our work has identified *DIN6* and *DIN11* as key regulators that support virus infections in systemically infected host plants, and outlines a number of important questions which need to be answered to elucidate the specific mechanisms and molecular pathways involving *DIN6* and *DIN11* activities during infections in plants.

EXPERIMENTAL PROCEDURES

Plant material, virus inoculation and sampling procedure

All Arabidopsis plants were in the Columbia background (Col-0), and were grown in controlled environment chambers under 16 h/8 h of light/dark at 19–22 °C. Homozygous plants for T-DNA insertion lines *din1* (NASC code N665464, SALK_020571C) and *din6* (NASC code N479505, GK-829B05) were used. Homozygosity of the mutant alleles was verified by PCR genotyping and by sequencing of the PCR products. *din1* contains a T-DNA insertion in the first intron of the *At4g35770* locus (genomic location 16 945 146). *din6* contains a T-DNA insertion in the first exon of the *At3g47340* gene (genomic location 17 441 009), 35 nucleotides downstream from the translation initiation site. The primers used for PCR genotyping of homozygous mutants are listed in Table S2 (see Supporting Information).

Nicotiana benthamiana plants were grown in controlled environment chambers under 16 h/8 h of light/dark at 25 °C, and were inoculated at approximately 21 days after germination by infiltration of *Agrobacterium tumefaciens* cultures containing TRV PpK20 cDNA clones of RNA1 (pTRV1)- and RNA2 (pTRV2)-derived constructs (mixed in a 1:1 ratio), or containing PVX (pgR107)-recombinant derivatives, as described previously (Liu *et al.*, 2002). Three-week-old Arabidopsis plants were inoculated with TRV using extract sap from systemically TRV-infected *N. benthamiana* leaves, as described previously (Donaire *et al.*, 2008). Mock inoculation was performed using sap from healthy, non-infected *N. benthamiana* leaves. Virus infection was corroborated by RT-PCR using TRV or PVX sequence-specific primers (Table S3, see Supporting Information).

Unless otherwise indicated, two or three newly emerging leaves per plant were collected from *N. benthamiana* infected with TRV or PVX derivatives used for VIGS at ~16 dai. Two or three upper non-inoculated rosette leaves per plant were collected from mock- and TRV-inoculated *Arabidopsis* at ~8 dai. Each sample consisted of RNA pooled from four to ten plants.

Construction of TRV- and PVX-derived plasmids

The pTRV1, pTRV2 and pgR107-PVX DNA vectors used in this study have been described previously (Carrington *et al.*, 1999; Liu *et al.*, 2002). To generate the recombinant TRV-GFP clone, the soluble modified GFP (smGFP) (Chiu *et al.*, 1996) gene cassette under the coat protein promoter of *Pea early-browning tobnavirus* (PEBV) was inserted into the multiple cloning site (MCS) of pTRV2. To generate the pTRV2 and pgR107 derivatives used for VIGS, cDNA fragments of NbNRIP1 (EU332891.1), AtDIN6/ASN1 (At3g47340) and NbDIN11 transcripts were RT-PCR amplified from RNA preparations and cloned into pCR2.1 vectors (Invitrogen, Barcelona, Spain) for DNA sequencing. A 303-bp fragment of *N. benthamiana* NbNRIP1, which corresponds to nucleotides 58–360, was amplified using NbNRIP1-F and NbNRIP1-R primers. A 306-bp fragment at nucleotide positions 25–330 of *Arabidopsis* AtDIN6/ASN1, which shares 77% sequence similarity at the nucleotide level with *N. benthamiana* NbASN (GQ354808/C9DFA8), was amplified using AtDIN6-F and AtDIN6-R primers. A 322-bp fragment, corresponding to nucleotides 462–784 of the *DIN11* homologue of *S. tuberosum* (clone 069B08), was amplified using StDIN11-F and StDIN11-R primers. A 606-bp fragment of the smGFP-protein coding sequence was amplified using GFP-F and GFP-R primers. cDNA fragments were excised from pCR2.1 vectors and inserted into the MCS of TRV2 or pgR107. All primers are listed in Table S3.

Generation of transgenic RNAi lines

The RNAi Gateway® vector pH 7GWIWG2 (II) (Invitrogen) was used to generate an RNAi construct targeted against AtDIN11. The selected target region comprised a 431-bp fragment from the 5' terminal region of the *Arabidopsis* AtDIN11 mRNA that was unique to that particular gene. The primers used are listed in Table S4 (see Supporting Information). All constructs were confirmed by DNA sequencing. The resulting plasmid was transformed into *A. tumefaciens* strain GV3101 by electroporation. *Arabidopsis* plants of the Col-0 ecotype were then transformed using the floral dipping method and selected on the basis of kanamycin resistance (Clough and Bent, 1998). Homozygous T3 transgenic lines were screened for transcript abundance by qRT-PCR, and only transgenic lines containing reduced levels of *DIN11* transcripts relative to the expression in the wild-type were used for virus infection assays.

RNA preparation and real-time qRT-PCR

Details on the minimum information for publication of real-time qPCR experiments are listed in Tables S5 and S6 (see Supporting Information) (Bustin *et al.*, 2009). To quantify the absolute copy number of TRV RNA1, a standard curve of known concentration of *in vitro* transcripts was used.

Absolute quantification of viral RNA1 was expressed as the number of viral copies per nanogram of total RNA. Sequence-specific primers are listed in Table S7 (see Supporting Information).

Protoplast preparation and transfection

Arabidopsis protoplasts were isolated from rosette leaves of 3-week-old plants and transiently transformed by the polyethylene glycol (PEG) method, as described previously (Yoo *et al.*, 2007), using 30 µg of purified pTRV1 plasmid DNA. Transfected protoplasts were cultured for 24 h in a growth chamber under 16 h of light at 20 °C and 8 h of dark at 22 °C. Protoplasts were harvested by centrifugation, suspended in 500 µL of Trizol reagent and stored at –80 °C for total RNA extraction.

Electrolyte leakage detection and chlorophyll quantification

Electrolyte leakage and chlorophyll *a* and *b* levels were measured in inoculated (TRV or mock) and upper non-inoculated leaves as described previously (De Leon *et al.*, 2002; Inskeep and Bloom, 1985).

Morphological trait analysis and fitness estimation

Measurements were taken at 16 and 29 dai from at least 30 plants per treatment. The weight and number of total seeds produced from healthy and TRV-infected *Arabidopsis* plants were measured from at least 30 individual plants per treatment.

Microarray data

CATMAs for gene expression profiling of TRV-infected *Arabidopsis* were conducted in our group as described previously (Crowe *et al.*, 2003; Fernandez-Calvino *et al.*, 2014; Rodrigo *et al.*, 2012), and deposited at the Gene Expression Omnibus (GEO) under accession numbers GSE15557/155562/15558.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 Analysis of senescence-associated traits during late *Tobacco rattle virus* (TRV) infection in *Arabidopsis*.

Fig. S2 Transcript accumulation of *Dark-inducible (DIN)* genes in response to *Tobacco rattle virus* (TRV) infection in *Arabidopsis*.

Fig. S3 Expression of defence-related genes in non-infected *DIN11* RNA interference (RNAi) lines.

Table S1 List of genes showing similar responsiveness to *Tobacco rattle virus* (TRV) infection, dark-induced senescence and age-dependent senescence.

Table S2 List of primers used for polymerase chain reaction (PCR) genotyping.

Table S3 List of primers used for virus amplification and in the construction of *Tobacco rattle virus* (TRV)-based virus-induced gene silencing (VIGS) vectors.

Table S4 List of primers used in the construction of *Dark-inducible (DIN)*-overexpressing *Arabidopsis* transgenic lines.

Table S5 Minimum information required for publication of quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) experiments involving messenger RNAs.

Table S6 Minimum information required for publication of quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) experiments involving absolute quantification of viral RNA.

Table S7 List of primers used in quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR).