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Differential expression of cucumber RNA-dependent RNA polymerase 1 genes during antiviral defence and resistance

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SUMMARY

RNA-dependent RNA polymerase 1 (RDR1) plays a crucial role in plant defence against viruses. In this study, it was observed that cucumber, *Cucumis sativus*, uniquely encodes a small gene family of four *RDR1* genes. The cucumber *RDR1* genes (*CsRDR1a*, *CsRDR1b* and duplicated *CsRDR1c1/c2*) shared 55%–60% homology in their encoded amino acid sequences. In healthy cucumber plants, *RDR1a* and *RDR1b* transcripts were expressed at higher levels than transcripts of *RDR1c1/c2*, which were barely detectable. The expression of all four *CsRDR1* genes was induced by virus infection, after which the expression level of *CsRDR1b* increased 10–20-fold in several virus-resistant cucumber cultivars and in a broad virus-resistant transgenic cucumber line expressing a high level of transgene small RNAs, all without alteration in salicylic acid (SA) levels. By comparison, *CsRDR1c1/c2* genes were highly induced (25–1300-fold) in susceptible cucumber cultivars infected with RNA or DNA viruses. Inhibition of *RDR1c1*/c2 expression led to increased virus accumulation. Ectopic application of SA induced the expression of cucumber *RDR1a*, *RDR1b* and *RDRc1/c2* genes. A constitutive high level of *RDR1b* gene expression independent of SA was found to be associated with broad virus resistance. These findings show that multiple *RDR1* genes are involved in virus resistance in cucumber and are regulated in a coordinated fashion with different expression profiles.

Keywords: cucumber, gene expression, RDR1, silencing, virus resistance.

INTRODUCTION

RNA silencing is a general eukaryotic cellular defence mechanism which regulates transcriptional or post-transcriptional gene expression in a sequence-specific manner by small interfering RNA (siRNA) molecules (Baulcombe, 2004; Dalmay *et al.*, 2000; Ding and Voinnet, 2007; Meister and Tuschl, 2004; Waterhouse *et al.*, 2001). In plants, the initial signals for the activation of gene silencing are exogenous (e.g. RNA virus) and endogenous (transposon) double-stranded RNA (dsRNA) molecules (Agrawal *et al.*, 2003; Hamilton and Baulcombe, 1999). RNA-dependent RNA polymerases (RDRs) are key regulators of RNA and virus silencing via the synthesis of dsRNAs that activate gene silencing after processing by DICER-like nucleases (DCLs) (Garcia-Ruiz *et al.*, 2010; Qi *et al.*, 2009; Qu, 2010; Qu *et al.*, 2008; Schiebel *et al.*, 1993; Xie *et al.*, 2001). Recently, it has been shown that the tomato genes for resistance against *Tomato yellow leaf curl virus*, designated *Ty-1* and *Ty-3*, are *RDR* genes (Verlaan *et al.*, 2013).

RDR1 is mainly associated with the antiviral RNA silencing pathway via the production and amplification of exogenous viral dsRNA in infected plants, which are subsequently digested by DCL-4 and DCL-2 into 21-nucleotide siRNA duplexes (Diaz-Pendon *et al.*, 2007; Qi *et al.*, 2009; Wang *et al.*, 2010). RDR1, RDR6 and, to some extent, RDR2 are associated with defence against viral RNA accumulation (Donaire *et al.*, 2008; Garcia-Ruiz *et al.*, 2010; Qi *et al.*, 2009; Wang *et al.*, 2010). RDR1 and RDR6 can be coordinated in defence against viruses, as demonstrated with *Cucumber mosaic virus* (CMV) in Arabidopsis, but seem to be antagonistic in *Plum pox virus* in transgenic *Nicotiana benthamiana* Domin expressing *NtRDR1* (Ying *et al.*, 2010).

The silencing of *RDR1* enhances the infection of several viruses in *Nicotiana tabacum* L., Arabidopsis and rice (Alamillo *et al.*, 2006; Rakhshandehroo *et al.*, 2009; Wang *et al.*, 2016; Xie *et al.*, 2001; Yu *et al.*, 2003). Furthermore, the absence of a functional RDR1 in *N. benthamiana* can explain the enhanced susceptibility of this species to many plant viruses (Yang *et al.*, 2004). However, suppression of *RDR1* in potato is not associated with increased virus susceptibility (Hunter *et al.*, 2016). By contrast, the constitutive expression of *RDR1*, observed in a transgenic cucumber Line 823 (*Cucumis sativus* L.), causes broad potyvirus resistance

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(Leibman *et al*., 2011). The regulation of *RDR1* following virus infection is not completely understood. Xie *et al*. (2001) demonstrated that either *Tobacco mosaic virus* infection or salicylic acid (SA) application induced RDR1 activity. Subsequently, this phenomenon was shown in different plant hosts for several viral pathogens and fungi, which implicates an important role for RDR1 in plant antiviral defence and against other biotic and abiotic stresses (Gilliland *et al*., 2003; Hunter *et al*., 2013; Liu *et al*., 2009; Yang *et al*., 2004; Yu *et al*., 2003). Several studies have shown the involvement of *RDR1* in the regulation of plant defences, e.g. phytohormone biosynthesis genes that mediate herbivore protection (Pandey *et al*., 2008), cuticular wax deposition (Lam *et al*., 2012; Pandey *et al*., 2008) and several other defence-related genes (Hunter *et al*., 2013; Rakhshandehroo *et al*., 2009). In addition, it has been shown that rice *RDR1* plays a role in gene regulation, and the null mutant *Osrdr1* shows alterations of small RNA (sRNA) accumulation and specific alterations of DNA methylation (Wang *et al*., 2014). *RDR1* is associated with DNA methylation in tomato, rice and Arabidopsis (Stroud *et al*., 2013; Wang *et al*., 2014). Recently, it has been shown that plant viruses induce microRNA (miRNA) expression, which regulates *RDR1* expression in rice via a molecular cascade (Wang *et al*., 2016). In addition, the biogenesis of viral-activated siRNAs has been demonstrated from the coding region of many host genes by *RDR1* in response to virus infection (Cao *et al*., 2014).

Here, we characterize a unique *RDR1* family of four genes in cucumber and their responses to virus infection. We show that, in cucumber, *RDR1b* is constitutively expressed at a high level only in resistant plants, whereas *RDR1c1* and *RDR1c2* are barely expressed in healthy plants, but induced to very high levels by RNA and DNA virus infection. The inhibition of cucumber *RDR1c1/2* leads to increased virus accumulation, demonstrating a role for these inducible genes in the control of virus infection. Multiple *RDR1* genes are involved in virus resistance in cucumber and are regulated in a coordinated fashion with different expression profiles. These *RDR1* genes may have either distinct or overlapping functions.

RESULTS

The *RDR1* gene family in cucumber

The *RDR1* sequences of several plant families were used to identify *Cucumis* spp. *RDR1* genes in the Cucurbit Genomics Database. We identified a number of putative *RDR1* genes within the *RDRα* clade (Willmann *et al*., 2011) of *Cucumis* spp. (Fig. 1). In cucumber, four putative unique *RDR1* family genes were identified in addition to *CsRDR2* and *CsRDR6* (Figs 1 and S1, Table S1, see Supporting Information). The *CsRDR1a* and *CsRDR1b* genes were positioned very close to each other (approximately 2.5 kbp apart) in a head-to-head orientation (http://www.icugi.org/cgi-bin/gb2/gbrowse/cucumber_v2/?name=gene:Csa5G239140). The *CsRDR1c1* and *CsRDR1c2* genes were within approximately 350 kbp of each other. The four exons of the *CsRDR1a*, *CsRDR1b*, *CsRDR1c1* and *CsRDR1c2* genes were similarly organized and, in each gene, similar exons were of comparable size (Fig. 1). This may indicate an ancestral *RDR1* for all *RDR1* genes in *Cucumis* spp.

The *CsRDR1a*, *CsRDR1b*, *CsRDR1c1* and *CsRDR1c2* genes were all expressed, but the expression of *CsRDR1c1* and *CsRD1c2* was scarcely detected in healthy plants under our conditions, compared with the high gene expression levels in virus-infected plants (Figs 2, S1 and S2, see Supporting Information). The coding regions of the *CsRDR1a*, *CsRDR1b*, *CsRDR1c1* and *CsRDR1c2* genes of cucumber cv. 'Shimshon' [resistant to CMV, *Cucumber green mottle mosaic virus* (CGMMV) and *Cucumber vein yellow virus* (CVYV)] and susceptible cv. 'Bet-Alfa' were cloned, sequenced and mapped to an *RDR1* cluster based on sequence homology (Fig. S3, see Supporting Information). There were no sequence differences between the *CsRDR1* genes of cv. 'Shimshon' and the corresponding genes in cv. 'Bet-Alfa' (data not shown). This was also the case for the putative promoter regions (approximately 3 kbp upstream of the coding region) of the four *RDR1* genes in cv. 'Shimshon' vs. the corresponding putative promoter regions in cv. 'Bet-Alfa' (data not shown). However, within a cultivar, the putative promoter regions of the *RDR1a*, *RDR1b* and *RDR1c* genes did not exhibit sequence similarity with each other, but the putative promoter regions of *RDR1c1* and *RDR1c2* were identical (data not shown).

The coding regions of the four *CsRDR1* genes from cucumber were compared with regard to their putative protein sequence (Fig. S3). The RNA binding domain and the catalytic motif 'DLDGD' were found in all RDR1 sequences (Fig. S3). The amino acid alignment of the putative cucumber RDR1a, RDR1b, RDR1c1 and RDR1c2 exhibited 55%–60% homology. RDR1a and RDR1b exhibited 60% homology, and both showed *c*. 55% homology to RDR1c1 and RDRc2. The latter pair shared 98% sequence homology to each other.

Phylogenetic analysis was performed on the four putative cucumber *CsRDR1*s and *RDR1* sequences from other cucurbit species [*Citrullus lanatus* (Thunb.) Matsum. & Nakai (watermelon) and *Cucumis melo* (muskmelon) (XN_008467032)], together with *RDR*s from other selected species (Fig. 3). The three types of *CsRDR1* clustered into separate groups. *CsRDR1a* clustered together with melon and watermelon *RDR1a* homologues. *CsRDR1b* and *CsRDR1c* clustered with their watermelon homologues, respectively, in two separate groups. The analysis showed that *CsRDR1b* was more related to *CsRDR1a* than to *CsRDR1c1* or *CsRDR1c2*. *CsRDR1b* was closely related to watermelon *ClRDR1b*, whereas *CsRDR1c1* and *CsRDR1c2* clustered together with watermelon *ClRDR1c*. The latter were more closely related to the *Hordeum vulgare* L. and Solanaceae *RDR1* (*Solanum lycopersicum* L.

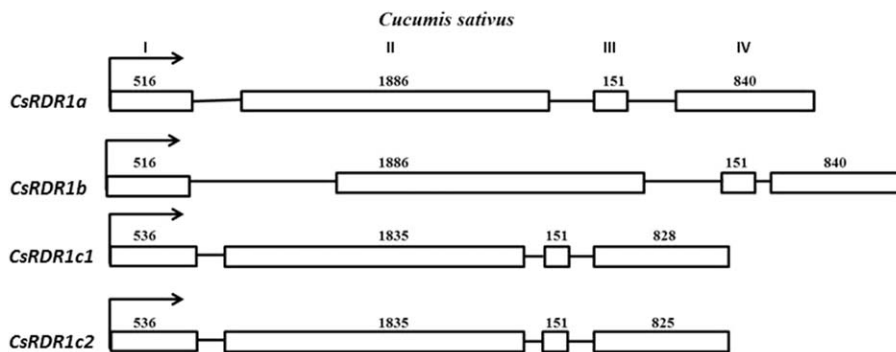

Fig. 1 Genome maps of *CsRDR1a*, *CsRDR1b*, *CsRDR1c1* and *CsRDR1c2* genes of cucumber (*Cucumis sativus*). Boxes represent exons and lines indicate introns. The genome maps were based on the cucumber Genomics Database (http://www.icugi.org). The numbers indicate the size of the exons (in nucleotides) and the start codon (ATG) is represented by an arrow. Exon numbers are marked in Roman numerals (I–IV).

and *Nicotiana tabacum*) than to *A. thaliana* (L.) Heynh. *AtRDR1* was more closely related to *CsRDR1b* and *ClRDR1b* than to any other *RDR1* (Fig. 3).

Participation of *CsRDR1* genes in virus resistance

We have shown previously that there is an association between multiple virus resistance and increased *CsRDR1* gene expression (Leibman *et al.*, 2011). Homozygous transgenic cucumber lines 823 and 887, expressing a *Zucchini yellow mosaic virus* (ZYMV) dsRNA fragment of the HCPro coding region in cv. 'Ilan', exhibited 'immunity' to ZYMV infection. Interestingly, only line 823 showed broad potyvirus resistance to ZYMV, *Watermelon mosaic virus* (immunity) and *Papaya ringspot virus* (tolerance), associated with a significant increase in *CsRDR1* gene expression (Leibman *et al.*, 2011). At the time of the study by Leibman *et al.* (2011), the existence of the cucumber *RDR1* gene family was unknown. To determine which of the four *CsRDR1* genes was associated with this resistance in line 823, but not line 887, we examined the expression of all *CsRDR1*s in the same cultivars and homozygous transgenic lines (Fig. 2).

The expression level of *CsRDR1a* in healthy cucumber leaves was higher in the resistant transgenic lines 823 and 887 and in cv. 'Shimshon' (about 2.3–4.1-fold) than in the virus-susceptible cvs. 'Ilan' and 'Bet-Alfa' (Fig. 2). Substantial differences (approximately 10–14-fold) were observed in the *CsRDR1b* expression level in the healthy plants of resistant cv. 'Shimshon' and transgenic line 823 compared with healthy plants of the susceptible cvs. 'Bet-Alfa' and 'Ilan' and transgenic line 887 (Fig. 2). The constitutive *CsRDR1b* expression levels in healthy virus-resistant cultivars indicate a possible association of the *CsRDR1b* expression level with broad virus resistance – 'like a resistance gene'. The expression levels of *CsRDR1c1* and *CsRDR1c2* (combined in Fig. 2 as *RDR1c*) remained very low in leaves and roots, and no significant differences could be seen between the resistant and susceptible cultivars or the transgenic lines (Figs 2, S2 and S4, see Supporting Information). We compared the expression level of *CsRDR1c1* and *CsRDR1c2* in ZYMV- and CMV-infected leaves of the resistant cv. 'Shimshon' and susceptible cv. 'Bet-Alfa' cucumbers (Fig. S2). In both cultivars, the expression of *CsRDR1c1* and *CsRDR1c2* was highly induced compared with the barely detectable mRNA levels in healthy plants (Figs S1, S2 vs. Fig. 2). These data indicated that the expression levels of *CsRDR1c1* and *CsRDR1c2* had similar kinetics and response to virus infection, and therefore, in the following experiments, the expression level of *CsRDR1c1* + *CsRDR1c2* was measured together, and is given as *CsRDR1c*.

To determine whether virus accumulation was affected by higher basal levels of expression of the *CsRDR1* gene family, resistance was determined for three RNA viruses of different families (Fig. 4): CMV, a member of the genus *Cucumovirus*, family *Bromoviridae*; CGMMV, a member of the genus *Tobamovirus*, family *Virgaviridae*; and CVYV, a member of the genus *Ipomovirus*, family *Potyviridae*. These results were compared with the responses to infection by ZYMV, a member of the genus *Potyvirus*, family *Potyviridae* (Leibman *et al.*, 2011). Resistance was determined by symptom expression and viral RNA accumulation was measured by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) at various days post-inoculation (dpi) (Fig. 4). Infection of cv. 'Shimshon' and transgenic line 823 with CMV and CVYV did not induce symptoms, whereas CGMMV induced very mild symptoms at 14 dpi. However, CMV and CGMMV infection caused severe symptom development in cvs. 'Bet-Alfa', 'Ilan' and transgenic line 887 (Fig. 4). CVYV infection caused severe symptoms in cv. 'Bet-Alfa' and mild chlorotic lesions in cv. 'Ilan' and transgenic line 887 (Fig. 4). The asymptomatic phenotype in cv. 'Shimshon' and line 823 was correlated with the low viral RNA accumulation (Fig. 4), i.e. tolerance. At 7 dpi, CMV RNAs accumulated to approximately 10-fold greater levels in cv. 'Bet-Alfa' relative to cv. 'Ilan' and line 887, but were undetectable in cv. 'Shimshon' and line 823. Significantly higher levels of CMV RNAs accumulated at 21 dpi in cvs. 'Bet-Alfa', 'Ilan' and line 887 compared with cv. 'Shimshon' and line 823 (Fig. 4). Similarly, significantly higher levels of accumulation of CGMMV and CVYV RNAs, at 7 and 14 dpi, respectively, were observed in cv. 'Bet-Alfa' and transgenic line 887 compared with 'Shimshon' and transgenic line 823 (Fig. 4). Differences in RNA accumulation following

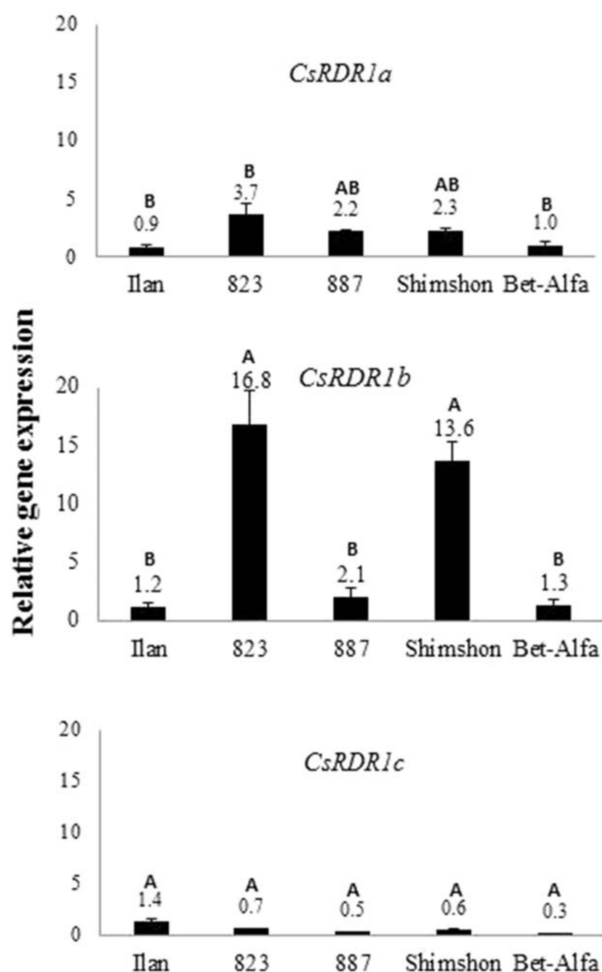

Fig. 2 Expression levels of *CsRDR1* genes (*CsRDR1a*, *CsRDR1b* and *CsRDR1c* (*CsRDR1c1* + *CsRDR1c2* together) in non-inoculated cucumber plants. Gene expression analysis of *CsRDR1a*, *CsRDR1b* and *CsRDR1c* genes of virus-susceptible 'Bet-Alfa' and 'Ilan', *Cucumber mosaic virus* (CMV)-, *Cucumber green mottle mosaic virus* (CGMMV)- and *Cucumber vein yellow virus* (CVYV)-resistant 'Shimshon', transgenic cucumber line 823 [*Zucchini yellow mosaic virus* (ZYMV) (resistant), *Watermelon mosaic virus* (resistant) and *Papaya ringspot virus* (tolerant)] and line 887 (ZYMV resistant). Total RNAs were extracted from leaves of each genotype and the relative expression levels of each gene were determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and were calculated using the $\Delta\Delta Ct$ method normalized to *Fbox* gene expression levels. Each bar is the mean of three replicates, each of three plants. The error bars denote standard deviations, and the different letters above the bars indicate statistically significant differences between the investigated transgenic lines and cultivars ($P < 0.01$).

inoculation with CVYV were much more pronounced between susceptible (cvs. 'Ilan', 'Bet-Alfa' and Line 887) and resistant (cv. 'Shimshon' and Line 823) plants at 14 dpi (Fig. 4). These results reinforce our previous observations with ZYMV (Leibman *et al.*, 2011), in which the resistance of transgenic line 823 was independent of the transgene sequence and was probably associated with the level of expression of *RDR1*.

Kinetics of expression of *CsRDR1* genes during virus infection

As *RDR1* gene expression is involved in resistance against plant viruses (Xie *et al.*, 2001; Yu *et al.*, 2003), mediated by RNA silencing (Cao *et al.*, 2014; Garcia-Ruiz *et al.*, 2010; Wang *et al.*, 2010), we assessed the expression levels of the *RDR1* genes in virus-infected plants. The expression levels of *CsRDR1a–c* in ZYMV-infected leaves of cucumber cv. 'Shimshon' and cvs. 'Bet-Alfa' and 'Ilan' (all susceptible to infection by ZYMV) (Fig. 5A) were compared. In all cultivars, significantly increased levels of *CsRDR1a* were observed at 11 dpi compared with healthy plants. However, earlier (4 and 7 dpi), no differences could be seen in the responses of cvs. 'Shimshon' and 'Bet-Alfa', although an increased level of *CsRDR1a* (approximately two-fold) was measured in cv. 'Ilan' at 7 dpi (Fig. 5A). *CsRDR1b* expression levels increased as a result of ZYMV infection at 7 and 11 dpi in cvs. 'Ilan' and 'Bet-Alfa' and at 11 dpi in cv. 'Shimshon' (Fig. 5A). It is important to note that the approximately eight-fold increase in the level of *CsRDR1b* expression in cv. 'Shimshon' at 11 dpi was in addition to the high *CsRDR1b* expression level observed in healthy plants of cv. 'Shimshon' (Fig. 5A).

The level of *CsRDR1c* gene expression increased dramatically in all cucumber cultivars after ZYMV infection (Fig. 5A): in cv. 'Bet-Alfa', the *CsRDR1c* level increased about four-fold at 4 dpi and by more than 400-fold at 7 and 11 dpi; in susceptible cv. 'Ilan', a >300-fold increase in *CsRDR1c* expression was observed at 7 and 11 dpi; and, in cv. 'Shimshon', a 122-fold increase in *RDR1c* expression was observed at 11 dpi. These data indicated that *CsRDR1c* expression was highly induced via ZYMV infection and was almost undetectable in healthy cucumber. An important question is whether the induction of *CsRDR1c* expression is a general phenomenon for cucumber infected with RNA and/or DNA viruses.

To examine this, the expression level of *CsRDR1c* was determined in cvs. 'Shimshon' and 'Bet-Alfa' at 14 dpi for RNA viruses (CMV, CGMMV and CVYV) and at 15 dpi for a DNA virus, the geminivirus *Squash leaf curl virus* (SLCV) (Fig. 5B). The expression of *RDR1c* was highly induced to different extents by all three RNA viruses and the geminivirus SLCV. A higher level of *RDR1c* induction was observed in cv. 'Bet-Alfa' infected with CVYV and CMV (approximately 600–700-fold). However, in cv. 'Shimshon' following infection by the same viruses (CVYV and CMV; Fig. 5B), the induction of *RDR1c* was rather less (3–90-fold) as a result of the resistance of cv. 'Shimshon' to these viruses (Figs 4 and 5B).

The increase in expression level of *RDR1c* in CGMMV-infected plants was lower (about 20-fold) when compared with infection with CMV, CVYV (Fig. 5B) and ZYMV (Fig. 5A). It has been shown that geminiviruses are subject to RNA silencing (Vanitharani *et al.*, 2003). Therefore, we tested the *CsRDR1c* expression level in cv. 'Bet-Alfa' infected with SLCV. The expression of *CsRDR1c* was induced about 20-fold in leaves infected with SLCV (Fig. 5B).

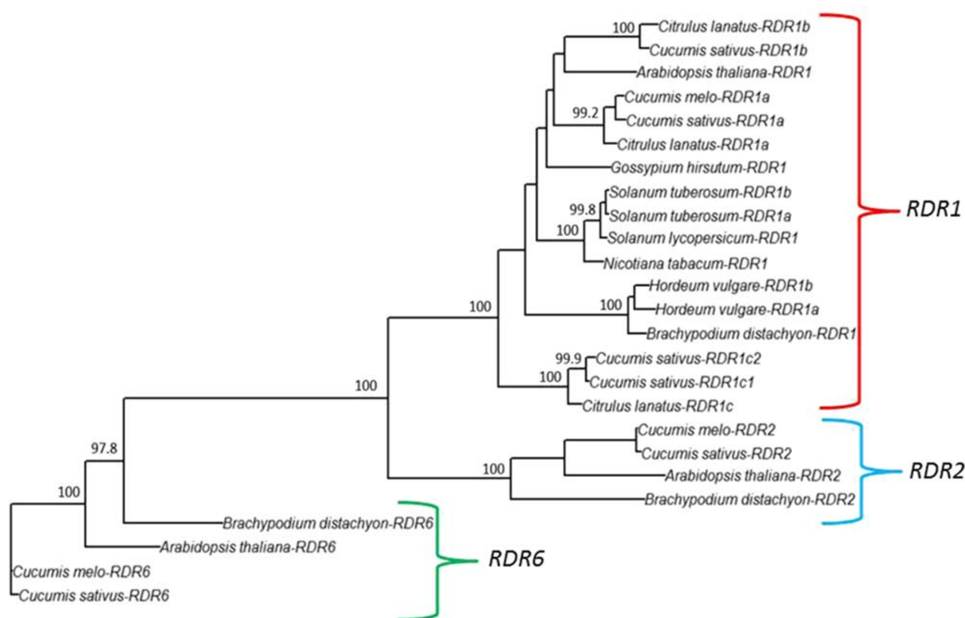

Fig. 3 Phylogenetic tree of RNA-dependent RNA polymerase α (RDRα) clade members. The phylogenetic tree was constructed from 25 proteins of 10 species using the maximum likelihood method implemented in 'phyml'. The analysis separated the genes into three distinct clades: *RDR1*, *RDR2* and *RDR6*. Values left of the internal nodes are the percentage of bootstrap resampling replicates (out of 100) that support the tree topology. Only bootstrap values of $\geq$90% and calculated distances are shown. The accession numbers of the RDR genes are listed in Table S1.

Interestingly, SLCV-infected cv. 'Bet-Alfa' plants showed mild symptoms at 2 weeks post-infection, from which the plants recovered.

Inhibition of *CsRDR1c* gene expression enhances virus accumulation

To determine whether *CsRDR1c* has a specific effect on virus systemic infection, we used ZYMV-based virus-induced gene silencing (VIGS) technology (Shoresh *et al*., 2006) to suppress the accumulation of *CsRDR1c* mRNA. A 250-bp *CsRDR1c* sequence was inserted into two engineered ZYMV strains: ZYMV^{FRNK} (severe) and ZYMV^{FINK} (symptomless) (Gal-On, 2007). ZYMV constructs with a *CsRDR1c* fragment (ZYMV-Rc and ZYMV-Ic) were infectious on cucumber and caused symptoms similar to those of the parental strains. Cucumber plants infected with recombinant viruses (ZYMV-Rc, ZYMV-Ic and ZYMV-wt) expressed similar levels of *CsRDR1a* and *CsRDR1b* (Fig. 6). However, the expression level of *CsRDR1c* was significantly decreased in cucumber leaves infected with ZYMV-Rc or ZYMV-Ic compared with infection with ZYMV-wt (Fig. 6). These data show that the *CsRDR1c* transcript level was partially silenced by the ZYMV-VIGS system (Fig. 6). The decrease in *CsRDR1c* expression levels accompanied a significant increase in the level of both recombinant viruses (5.2-fold with ZYMV-Rc and 3.9-fold with ZYMV-Ic), compared with ZYMV-wt, indicating the involvement of *CsRDR1c* in defence.

Signal transduction in Cs*RDR1c1* gene expression induced by virus infection

To examine whether a signal causes the induction of *CsRDR1* expression in a leaf prior to the appearance of virus from an inoculated lower leaf, a time course analysis of *CsRDR1b* and *CsRDR1c* gene expression was performed in a systemic leaf, following cotyledon inoculation of cucumber cv. 'Bet-Alfa' with CMV (Fig. S5, see Supporting Information). CMV systemic infection could not be detected at 24 and 48 h post-infection (hpi) in the first true leaf above the inoculated cotyledon. Low levels of CMV accumulation were detected in the first true leaf at 65 hpi, and subsequently very high levels of CMV could be measured in the systemically infected leaf at 5 and 7 dpi (Fig. S5). The expression levels of *CsRDR1b* increased by approximately two- and five-fold in virus-free, true leaves of inoculated plants at 24 and 48 hpi, respectively, and by approximately 3.5- and nine-fold in systemically infected leaves at 5 and 7 dpi, respectively (Fig. S5). *CsRDR1*c expression levels increased by about four-fold at 48 hpi in the virus-free true leaves of inoculated plants. However, *CsRDR1c* levels in the first systemically infected leaves increased by more than 1000-fold at 5 and 7 dpi. These data indicate that CMV infection induces a systemic signal that increases the expression of *CsRDR1b* and *CsRDR1c* prior to virus infection and that, in infected tissues, *CsRDR1c* expression is induced to very high levels.

Exogenous SA induces *RDR* expression in different species (Carr *et al*., 2010). We therefore tested the effect of SA on the expression levels of *CsRDR1a–c* in resistant cv. 'Shimshon' and susceptible cv. 'Bet-Alfa' cucumber. Ectopic application of SA for 6 days induced the expression of *CsRDR1a–c* in both cultivars (Fig. 7A). *CsRDR1a* and *CsRDR1b* levels in cv. 'Bet-Alfa' (six-fold increase for *RDR1a* and 15-fold increase for *RDR1b*) were approximately double those observed in cv. 'Shimshon' (three-fold for *RDR1a* and seven-fold for *RDR1b*), but a much higher induction was observed with *CsRDR1c*

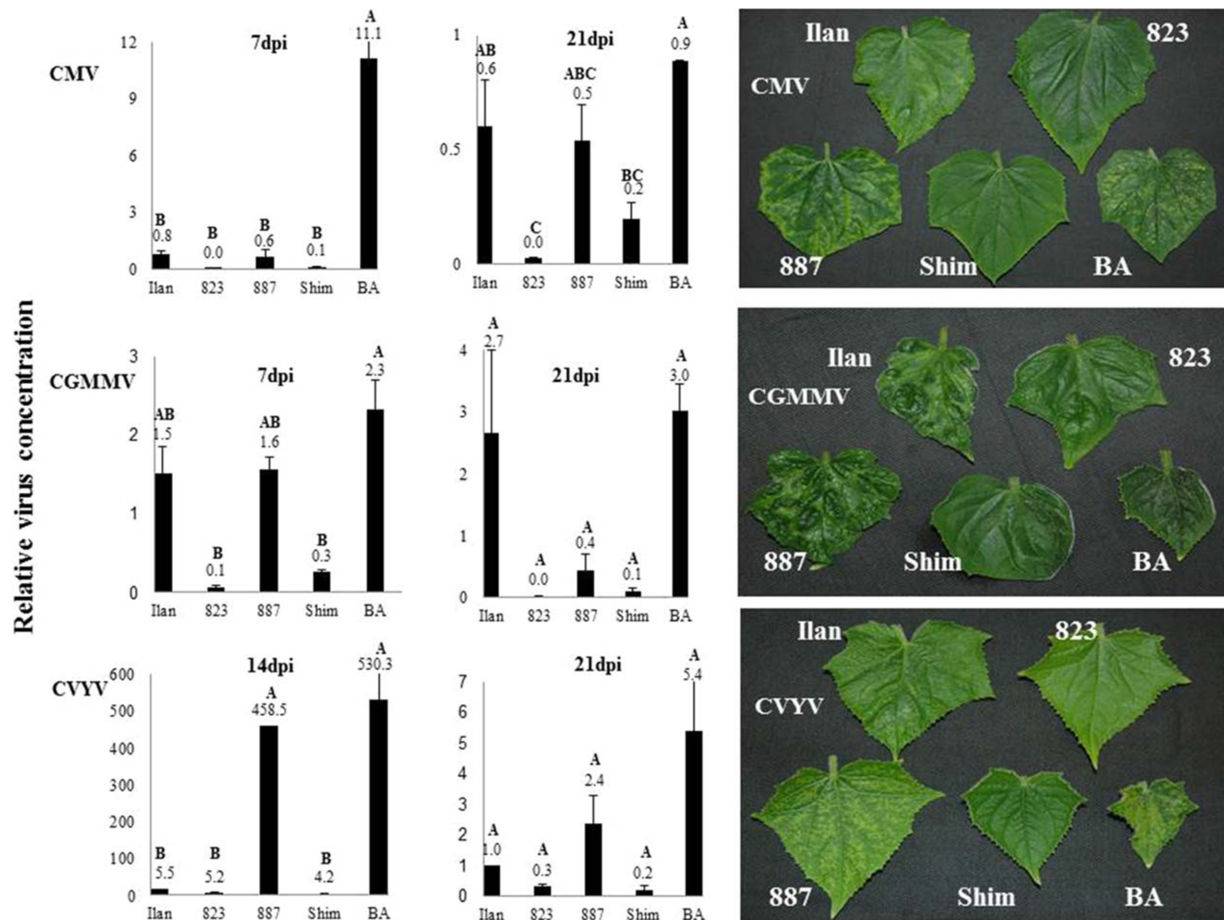

Fig. 4 Analysis of virus resistance of cucumber cultivars and transgenic lines to three virus families. The cultivars 'Bet-Alfa' (BA), 'Shimshon' (Shim) and 'Ilan', and transgenic lines 887 and 823, were sap inoculated with *Cucumber mosaic virus* (CMV), *Cucumber green mottle mosaic virus* (CGMMV) and *Cucumber vein yellow virus* (CVYV), and symptoms were recorded at 14–18 days post-inoculation (dpi) (right panels). Relative viral RNA accumulation was measured by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) (left panels) at different dpi, and the levels of each virus were calculated using the $\Delta\Delta Ct$ method normalized to *Fbox* gene expression levels. Each bar is the mean of three replicates, each of three plants. The error bars denote standard deviations, and the different letters above the bars indicate statistically significant differences between transgenic lines and cultivars ($P < 0.01$, except for $P < 0.05$ for CMV at 21 dpi).

in both cultivars (27-fold in 'Bet-Alfa' and 33-fold in 'Shimshon') (Fig. 7A). *CsRDR1a*, *CsRDR1b* and *CsRDR1c* expression by SA treatment decreased to initial levels at 7 days after spraying (Fig. 7B), which may indicate a transient effect of SA on the induction of *CsRDR1* gene expression (Fig. 7A).

We tested the expression of other genes in the SA signalling pathway in transgenic line 823 (with high endogenous *CsRDR1b* expression levels) and non-transgenic cv. 'Ilan' in the absence of added SA. Here, similar levels of expression were measured for the genes *SID2* and *EDS5* upstream of SA synthesis, and for the genes *WIN3* and *WRKY22* downstream of SA synthesis (Carr *et al*., 2010; Kumar and Klessig, 2000) (Fig. 7D). Similar accumulation levels of SA were measured in cvs. 'Bet-Alfa' and 'Shimshon', and lines 887 and 823 (Fig. 7C). These data indicate that the high endogenous level of expression of *CsRDR1b* is not caused by increased endogenous levels of SA or of various genes in the SA signalling pathway. Nevertheless, exogenous SA can increase the levels of expression of *CsRDR1b* still further (Fig. 7A).

DISCUSSION

Organization and expression of cucumber *RDR1* genes

Our studies reveal a unique small gene family of four *CsRDR1*s in cucumber, in addition to *CsRDR2* and *CsRDR6*. Four cucumber *RDR1* genes were identified (Figs 1 and 3). The cucumber *RDR1* genes can be separated into *1a*, *1b* and *1c* (*1c1* + *1c2*) based on sequence similarity and gene expression (Figs 2, S2 and S3). *CsRDR1c1* and *CsRDR1c2* are very similar (essentially copies), expressed to similar levels in different virus-infected cucumber cultivars (Fig. S2). Such a *RDR1* gene family (*RDR1a–c*), with members varying by 30%–40%, has not been demonstrated in other

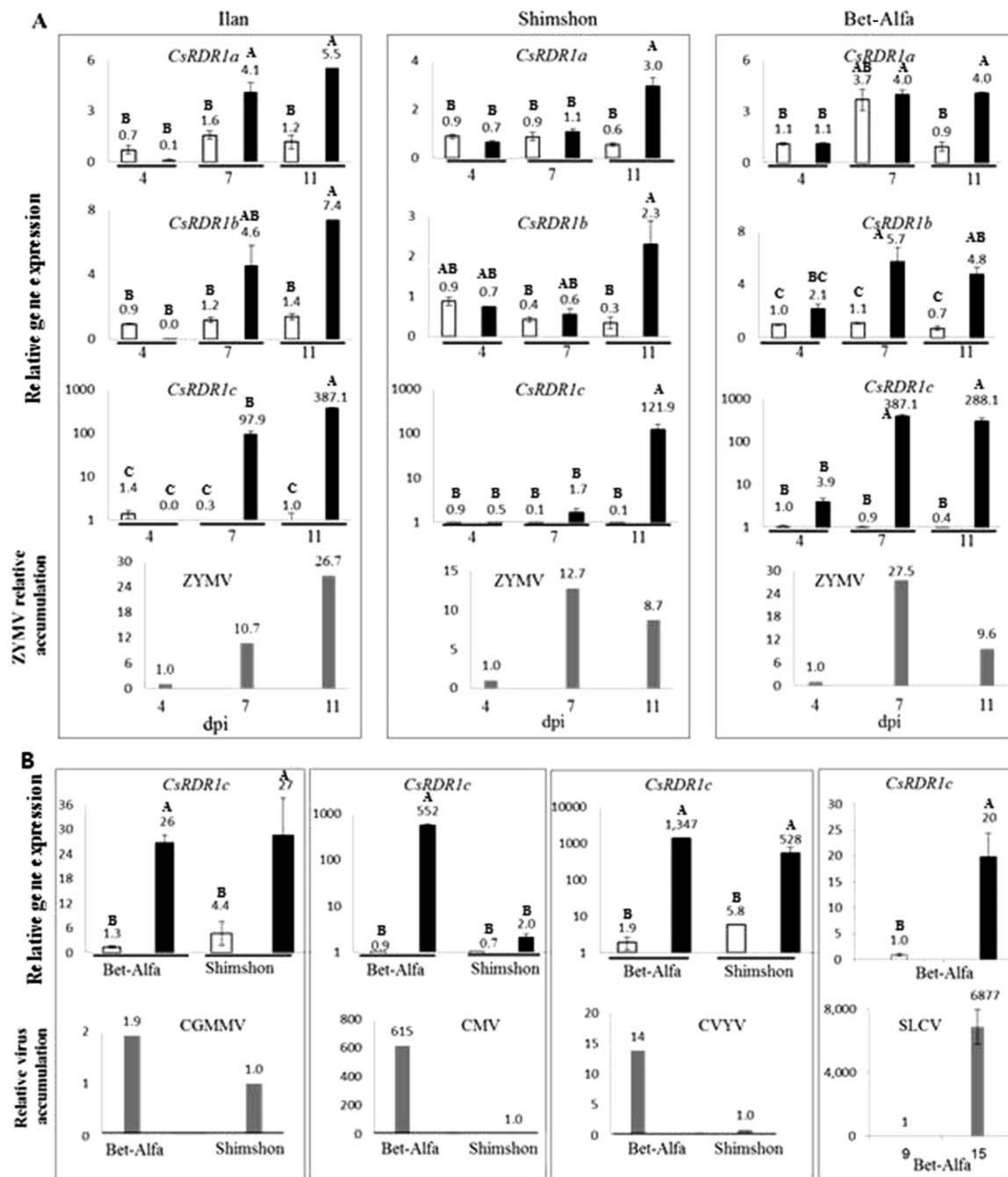

Fig. 5 The expression levels of the *RDR1* gene family in cucumber are affected by virus infection, and *CsRDR1c* is highly induced with RNA and DNA viruses. (A) *CsRDR1a*, *CsRDR1b* and *CsRDR1c* expression was analysed in healthy (white bars) and *Zucchini yellow mosaic virus* (ZYMV)-infected (black bars) cucumber leaves from 'Ilan', 'Shimshon' and 'Bet-Alfa'. The relative levels of ZYMV in infected cucumber are presented (grey bars). Total RNAs were extracted from plants at 4, 7 and 11 days post-inoculation (dpi) with ZYMV. (B) Relative expression levels of *CsRDR1c* and virus nucleic acids in cucumber 'Shimshon' (as a reference) and 'Bet-Alfa', healthy and infected with *Cucumber green mottle mosaic virus* (CGMMV), *Cucumber mosaic virus* (CMV), *Cucumber vein yellow virus* (CVYV) (14 dpi) and *Squash leaf curl virus* (SLCV). Total RNA was extracted from virus-infected (black bars) and healthy (white bars) cucumber plants at different days after sap inoculation. First-strand cDNAs were prepared with oligo-dT and specific CGMMV and CMV primers. Quantitative polymerase chain reaction (qPCR) was performed with appropriate primers for *RDR1* mRNA for the coat protein genes of the different viruses and for the host *Fbox* gene for normalization. The relative expression level of each gene was calculated using the $\Delta\Delta Ct$ method normalized to the *Fbox* gene expression level. DNA was extracted from infected (with SLCV) and healthy cucumber at 9 and 15 days after whitefly inoculation and qPCR was performed on 15 dpi samples (not infected as a reference). Each bar is the mean of three replicates of three plants. The error bars denote standard deviations, and the different letters above the bars indicate statistically significant differences between infected and non-infected cultivars ($P < 0.01$, except $P < 0.05$ for the level of *CsRDR1a* in Bet-Alfa infected with CMV).

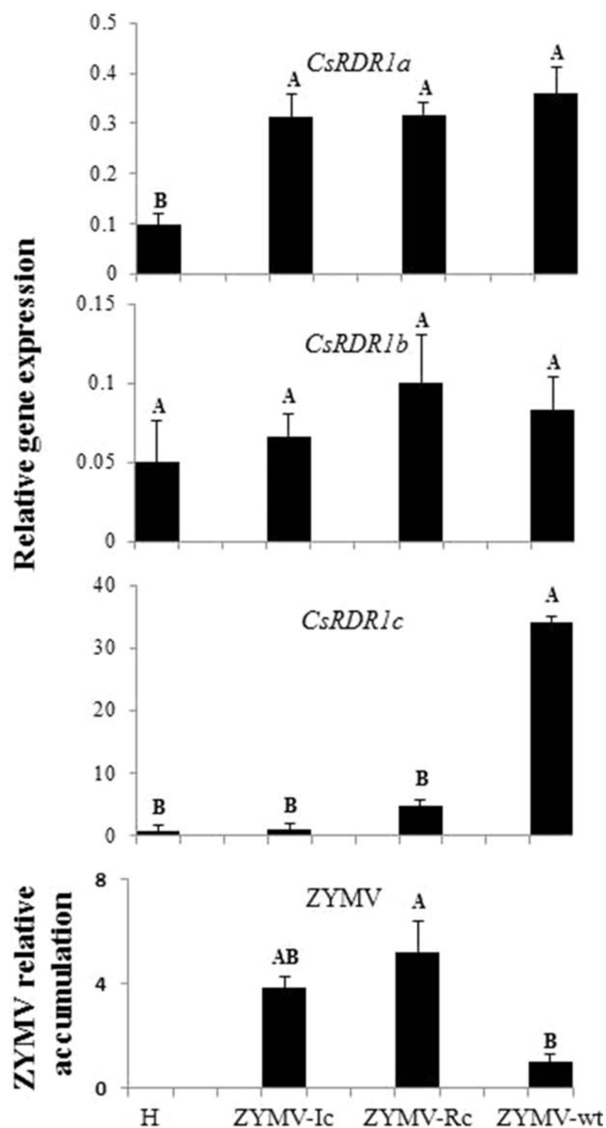

Fig. 6 Inhibition of *CsRDR1c* mRNA accumulation increases virus accumulation. The expression levels of *CsRDR1a*, *CsRDR1b* and *CsRDR1c* were examined in cucumber 'Bet-Alfa' infected with recombinant viruses ZYMV-Ic (symptomless) and ZYMV-Rc (severe) and with ZYMV-wt (severe). Total RNAs were extracted from plants at 7 days post-inoculation (dpi) and from healthy plants (H). First-strand cDNAs were prepared with oligo-dT and quantitative polymerase chain reaction (qPCR) was performed with the appropriate primers for *CsRDR1a*, *CsRDR1b*, *CsRDR1c* and the *Zucchini yellow mosaic virus* (ZYMV) coat protein (CP) gene. The relative expression level of each *CsRDR1* gene was calculated using the $\Delta\Delta Ct$ method normalized to *Fbox* gene expression. The relative virus titre is indicated in the bottom panel. Each bar is the mean of three replicates of three plants. The error bars denote standard deviations, and the different letters above the bars indicate statistically significant differences in *CsRDR1a*, *CsRDR1b* and *CsRDR1c* between infected plants and between the ZYMV titres ($P < 0.01$).

plant species. However, *RDR1* gene duplication (such as *RDR1c1* and *RDR1c2*, with members 98% identical) seems to be a common evolutionary phenomenon (Zong *et al.*, 2009), observed in both monocots (barley) (Madsen *et al.*, 2009) and other dicots (potato) (Hunter *et al.*, 2016). The unique *RDR1* family was also found in other Cucurbitaceae: *Citrullus* (watermelon; Cucurbit Genomics Database) (Fig. 3) and *Cucurbita* (squash, pumpkin; http://www.icugi.org/cgi-bin/ICuGI/index.cgi).

No differences were observed between each of the four *CsRDR1* genes plus their putative promoters (3 kb sequence upstream) in virus-susceptible cv. 'Bet-Alfa' compared with the same genes and putative promoters in multiple virus-resistant cv. 'Shimshon'. Thus, differences in *RDR1a–c* expression between these cultivars (Leibman *et al.*, 2011; Wang *et al.*, 2003) may be caused by modifications of enhancer elements, specific transcription factors that bind to these elements, epigenetic effects or regulators of *RDR1* gene expression, as demonstrated recently for the rice *RDR1* (Wang *et al.*, 2016). By contrast, the putative promoter sequences for *CsRDR1a–c* were different from each other (but with that of *RDR1c1* being the same as *RDR1c2*), indicating that regulation could differ at the promoter level, as demonstrated in Arabidopsis (Xu *et al.*, 2013).

Our gene cloning study enabled corrections of a situation which was previously unclear for this gene family. Previous interrogation of *CsRDR* in the Cucurbit Genomics Database identified five *RDR1* genes, labelled *CsRDR1a–e* (Gan *et al.*, 2016). In that *in silico* study, no *RDR1* genes were cloned and sequenced, in contrast with our study. Thus, *CsRDR1a* and *CsRDR1b* are the same in both studies, although *CsRDR1a* in the database contains a 100-bp deletion. In addition, *CsRDR1c* of Gan *et al.* (2016) has the same sequence as the 5′ half of *CsRDR1b* here, whereas *CsRDR1d* of Gan *et al.* (2016) has the same sequence as the 3′ half of our *CsRDR1c1*, and their *CsRDR1e* is the same as our *CsRDR1c2*.

Close phylogenetic relationships are notable between the *RDR1a* gene products of cucumber, melon and watermelon, as well as between the *RDR1b* and *RDR1c* gene products of cucumber and watermelon (Fig. 3). Such similarities suggest that the duplications leading to the accumulation of four *RDR1* genes, in close proximity on an arm of chromosome 5 of cucumber (Cucurbit Genomics Database), occurred before the separation of the ancestral cucurbits into modern taxa. In addition, the great diversity between the *CsRDR1a*, *CsRDR1b* and *CsRDR1c* genes (homology levels of 55%–60%), whilst retaining a much higher homology among the same genes from cucurbit species (Fig. 3), indicates that the genes evolved earlier to provide separate, although possibly overlapping, defence functions.

Kinetics of expression of *RDR1* genes during virus infection

CsRDR1a and *CsRDR1b* only showed moderate increases in gene expression as a result of ZYMV infection (2.5 to six-fold) in cvs. 'Ilan', 'Bet-Alfa' and 'Shimshon' (Fig. 5A). Notably, the increase in *CsRDR1b* levels in cv. 'Shimshon' was on top of the 13-fold higher constitutive level of expression found in healthy plants (Fig. 5A vs.

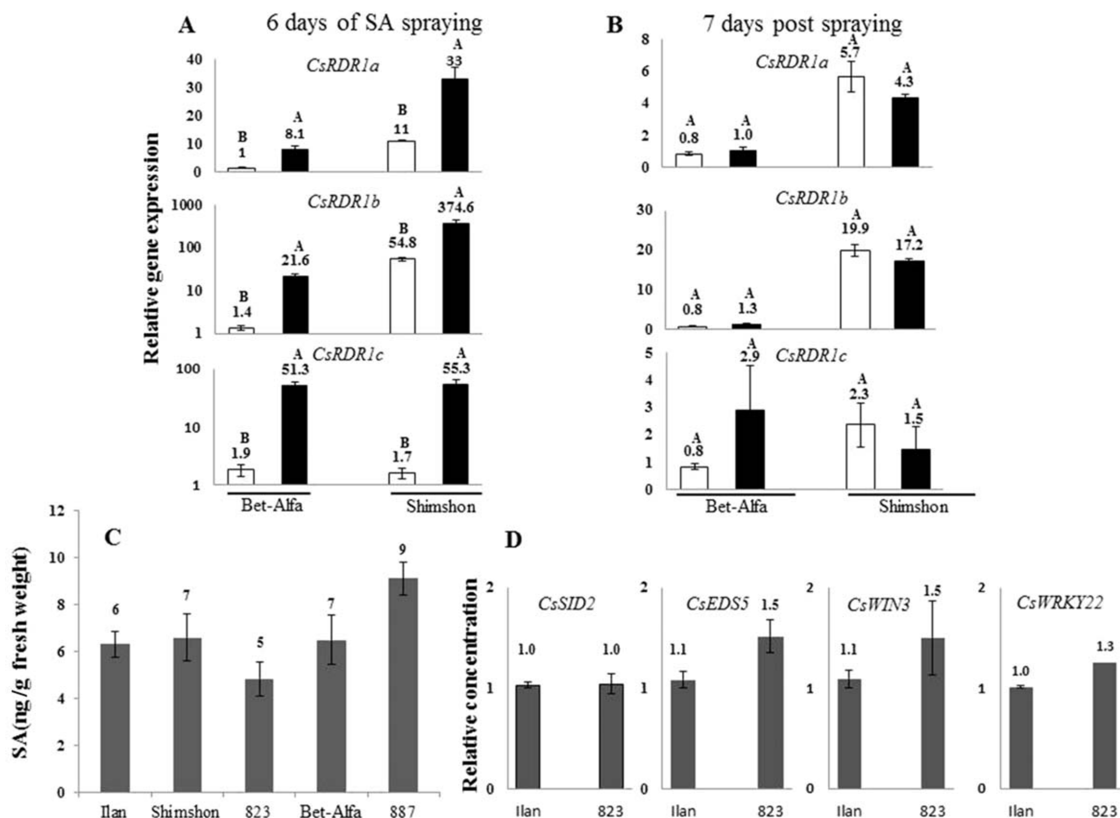

Fig. 7 The expression of *CsRDR1a*, *CsRDR1b* and *CsRDR1c* was induced by salicylic acid (SA). Expression levels of *CsRDR1a*, *CsRDR1b* and *CsRDR1c* in healthy cucumber ('Shimshon', 'Bet-Alfa') sprayed daily with a 1% solution of SA (black bars) and water control (white bars). Total RNAs were extracted from plants after spraying for 6 days (A) and following a 7-day interval after SA application (B). (C) SA content in cucumber 'Ilan', 'Bet-Alfa' and 'Shimshon', and transgenic lines 823 and 887. SA was extracted from three plants per cultivar and from the two transgenic lines at 14 days post-germination, and statistically significant differences between treatments were calculated by the MassLynx range test ($P < 0.05$). (D) Relative expression levels of *SID2*, *EDS5*, *WIN3* and *WRKY22* genes in healthy cv. Ilan and transgenic line 823. First-strand cDNAs were prepared from isolated mRNAs using oligo-dT, followed by quantitative polymerase chain reaction (qPCR) performed with appropriate primers for *CsRDR1a*, *CsRDR1b*, *CsRDR1c*, *SID2*, *EDS5*, *WIN3* and *WRKY22* genes, as well as for the *Fbox* gene used for normalization. The relative expression level of each gene was calculated using the $\Delta\Delta Ct$ method normalized to *Fbox* gene expression levels. Each bar is the mean of three replicates of three plants. The error bars denote standard deviations, and the different letters above the bars indicate statistically significant differences between SA treatments ($P < 0.01$). The statistical analysis in (A) was made separately for Bet-Alfa and Shimshon.

Fig. 2). However, *CsRDR1c* showed a much greater increase in expression after ZYMV infection, by several hundred-fold or more (11 dpi) in cvs. 'Ilan', 'Bet-Alfa' and 'Shimshon' (Fig. 5A).

A higher level of expression of *CsRDR1c* was also observed after infection by other viruses in cv. 'Bet-Alfa': 20-fold for CGMMV, 600-fold for CMV, 700-fold for CVYV and 20-fold for SLCV (Fig. 5B). Interestingly, in cv. 'Shimshon', which has a high endogenous *CsRDR1b* expression level, expression of *CsRDR1c* was only stimulated six-fold, three-fold and 90-fold following infection by CGMMV, CMV and CVYV, respectively (Fig. 5B). Cultivar 'Shimshon' shows good resistance to virus accumulation against all three viruses, but only moderate resistance against symptoms induced by CGMMV vs. good resistance to symptom development induced by CMV or CVYV (Figs 4 and 5B). Thus, the higher endogenous level of *CsRDR1b* may act as a new resistance gene, so that the reduced virus titre may obviate the need for strong *CsRDR1c* induction (Fig. 8). *CsRDR1c* plays a role in decreasing the virus titre, as demonstrated by the silencing of *CsRDR1c* expression using a ZYMV-VIGS vector (Fig. 6). Similarly, an *rdr1* rice mutant showed higher virus titre (Wang *et al*., 2016). Unfortunately, as ZYMV-mediated VIGS of the *CsRDR1a* and *CsRDR1b* genes was unsuccessful, we could not establish the effect of each gene on the expression of the other, or the effect of the silencing of each gene on the infection by different viruses.

The role of *CsRDR1a* in defence against virus infection is less clear, as it was induced only moderately, in line with the induction levels described for *RDR1* from plants that contain only a single *RDR1* (Alamillo *et al*., 2006; Rakhshandehroo *et al*., 2009; Xie *et al*., 2001; Yu *et al*., 2003), or two almost identical *RDR1*s (e.g. potato and barley) (Hunter *et al*., 2016; Madsen *et al*., 2009).

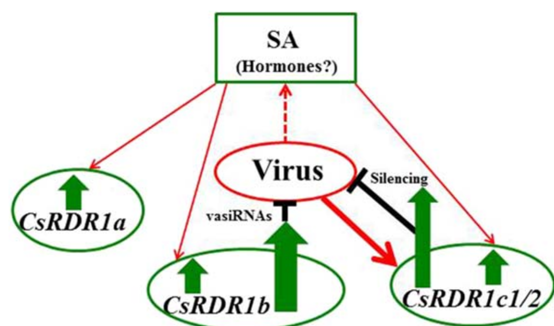

Fig. 8 A model describing the effects of virus infection on the expression of *CsRDR1* genes in susceptible and resistant plants. The model shows induced expression of the four *CsRDR1* genes (short, thick, green arrows) by salicylic acid (SA) (long thin red arrows) and possibly other phytohormones [jasmonic acid (JA) or abscisic acid (ABA)]. Virus-induced *CsRDR1* expression occurs via SA induction (red broken arrow). However, virus infection (thick red arrow) induces a much higher level of *CsRDR1c1/c2* (long, thick, green arrow) than does SA alone, leading to virus silencing. The high constitutively expressed *CsRDR1b* (thick green arrow) causes broad virus resistance. A high level of *RDR1b* expression is possibly associated with the production of viral-activated small interfering RNAs (vasiRNAs) (Cao *et al*., 2014), which activate broad-spectrum antiviral activity via widespread silencing of host genes.

Signalling responses in *RDR1* gene expression

Before virus could be detected in upper leaves, an increase in transcription of *CsRDR1b* and *CsRDR1c* genes was perceived (Fig. S5), suggesting that a signal, possibly SA, anticipated systemic virus appearance (Carr *et al*., 2010; Mayers *et al*., 2005; Zhu *et al*., 2014). Ectopic application of SA to cucumber leaves induced the expression of all four *CsRDR1* genes, especially *CsRDR1c* (Fig. 7A), as shown previously for *NtRDR1* and *AtRDR1* (Xie *et al*., 2001; Yu *et al*., 2003). However, a high endogenous level of *CsRDR1b* gene expression in broad virus-resistant lines (line 823 and cv. 'Shimshon') was not caused either by a higher basal level of SA (Fig. 7C) or increased activity of SA-mediated defence pathway genes (Fig. 7D). The cause of the association of a constitutively high *RDR1b* level and broad virus resistance remains unclear. Potential effects of other phytohormones or transcriptional regulator(s) on *CsRDR1b* gene expression cannot be dismissed. In addition, we assume that, in transgenic line 823, unlike in cv. 'Shimshon', the high level of *CsRDR1b* expression probably depends on the very high level of transgene-dsRNA/transgene-siRNA accumulation (Leibman *et al*., 2011)

Virus resistance and susceptibility in cucumber

The combined data on the effects of virus and signalling responses on the expression of *CsRDR1* genes are summarized in a model (Fig. 8) as follows: virus infection results in a weak induction of *CsRDR1a* and *CsRDR1b* (probably mediated by SA), but a strong induction of *CsRDR1c*. The strong induction of *CsRDR1c* by virus infection is much greater than that observed by application of SA alone (Figs 5, S2 and S5 vs. Fig. 7), and thus viruses may induce *CsRDR1c* by another pathway, possibly mediated by other phytohormones to which *RDR1* genes have been shown to respond (Hunter *et al*., 2013; Liu *et al*., 2009; Pandey and Baldwin, 2007; Xu *et al*., 2013). In addition, *RDR1c* induction could be mediated by either miRNA (Wang *et al*., 2016) or transcription factors via virus-associated siRNA (Cao *et al*., 2014). The higher level of induced *CsRDR1c* had an effect on virus accumulation, as demonstrated by gene silencing. In one cucumber cultivar ('Shimson') and a transgenic line (823), *CsRDR1b* was expressed constitutively at a high level (Figs 2 and 7). These plants are either tolerant or highly resistant to virus infection (Figs 4 and 5B). The constitutively high *CsRDR1b* transcript level strongly associated with virus resistance is independent of virus infection and endogenous SA level (Figs 2 and 7).

Overall, our study showed that multiple *RDR1* genes are involved in virus resistance in cucumber, are regulated in a coordinated fashion with different expression profiles, and may have either distinct or overlapping functions.

EXPERIMENTAL PROCEDURES

Plants, pathogens and inoculations

Three accessions of Bet-Alfa-type cucumbers, *Cucumis sativus*, were used for the biological and molecular analyses. These were the original 'Bet-Alfa' (Paris *et al*., 2012), as well as 'Shimshon', 'Ilan' (Zera'im Gedera Co., Gedera, Israel) and the homozygotic transgenic lines 823 and 887 (Leibman *et al*., 2011). Seeds were planted in a soil mixture and grown in a growth chamber under continuous white fluorescent light at 25 °C. Mechanical inoculation of cucumber seedlings with CMV, ZYMV, CGMMV (Table S2, see Supporting Information) and recombinant viruses ZYMV-Ic and ZYMV-Rc was performed according to Leibman *et al*. (2011). Inoculation of cucumber with CVYV and SLCV was performed by viruliferous whiteflies (Sufrin-Ringwald and Lapidot, 2011).

All experiments were sampled in the same manner for molecular analysis of RNA, DNA, endogenous genes and virus accumulation. Nine plants in groups of three (i.e. three replicates) were used per sample. Two 8-mm leaf discs were sampled per plant. Therefore, each repeat was a pool of six leaf discs from three plants.

Phylogenetic analysis

Protein sequences derived from three *RDR*α clade members (*RDR1*, *RDR2* and *RDR6*) from nine different species were aligned with the MAFFT program. Multiple sequence alignment (MSA) was performed globally for all pairs with maximum iterations of 1000. The robustness of the MSA was estimated with guidance2 (Sela *et al*., 2015) to be 0.971, which was slightly more robust than the local pair option. Furthermore, one MSA site with a confidence value of <0.25 was removed for downstream analysis. The phylogenetic tree was constructed using the likelihood method by the 'phyml' program (Guindon and Gascuel, 2003; Shimodaira and Hasegawa, 2001)

with the JTT substitution model. The robustness and confidence of tree nodes were estimated as a percentage of 1000 bootstrap resampling replicates

DNA and RNA extraction and PCR and qRT-PCR analysis

Total genomic DNA was extracted from cucumber leaves (approximately 40 mg) (two leaf discs per plant) by the method of Dellaporta *et al.* (1983). Diluted DNA (1 : 25) was used for PCR, with the appropriate primers for *RDR1* (*RDR1a*, *RDR1b*, *RDR1c1* and *RDR1c2*), *RDR2*, *RDR6* and F-box genes (Table S3, see Supporting Information). PCR conditions were the same as those described in Leibman *et al.* (2011).

RNA expression of endogenous genes and virus accumulation were determined by RT-PCR and qRT-PCR according to Leibman *et al.* (2011). RNA samples were collected from the second and third leaves of cucumber, and from whole roots. Total RNAs were extracted by a TRI-REAGENT kit (Molecular Research Center, Inc., Cincinnati, OH, USA) and adjusted to the same concentration prior to RT-PCR using a NanoDrop ND1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). First-strand cDNA was synthesized from 2 μg of total RNA using the Verso™ cDNA Kit (Thermo Fisher Scientific, Epsom, UK) with oligo(dT) primer (100 pmol) and specific virus reverse primers for CMV and CGMMV analysis. The cDNA was used for PCR and qPCR as described above. qPCRs were performed according to Leibman *et al.* (2011).

Construction of the ZYMV-VIGS vector

ZYMV^{FRNK} and ZYMV^{FINK} are potyvirus-based vectors for the expression of foreign genes and gene silencing in cucurbits (Arazi *et al.*, 2001; Shoresh *et al.*, 2006). To silence *RDR1c*, two ZYMV constructs were made which contained an *RDR1c* fragment: ZYMV-Rc and ZYMV-Ic. The *RDR1c* 225-bp fragment was amplified from *CsRDR1c* cDNA clones by PCR (Arazi *et al.*, 2001; Table S3).

Cloning and sequencing

RDR1a, *RDR1b*, *RDR1c1* and *RDR1c2* were cloned from cucumbers 'Bet-Alfa' and 'Shimshon' using the Cucurbit Genomics Database (http://www.icugi.org/). The intact *RDR1* mRNAs of cucumber were cloned with primers corresponding to the coding regions (Table S3). RT-PCR was performed as above and PCRs were performed using Phusion high-fidelity DNA polymerase and the manufacturer's protocol (New England BioLabs, Hitchin, UK). Fragments were cloned into pJet1.2/blunt Vector (Thermo Scientific) and sequenced twice.

Endogenous cucumber genes (homologous to Arabidopsis *WRKY22*, *EDS5*, *WIN3* and *SID3*) were cloned based on the Cucurbit Genomics Database and using the primers listed in Table S3. F-box genes were used for gene expression controls in cucumber (Leibman *et al.*, 2011). The expression of the endogenous genes *SID2*, *EDS5*, *WIN3* and *WRKY22* was analysed using specific primers (Table S3) from the Cucurbit Genomics Database.

SA sample preparation and analysis

Samples for SA analysis were extracted from three separate plants of each accession: 'Bet-Alfa', 'Shimshon' and transgenic lines 887 and 823. Leaf samples (250 mg per leaf) were taken from plants at 14 days post-germination, frozen in liquid N_2 and ground to a powder with a mortar and pestle. The powder was mixed with 750 μL MeOH–H_2O–HOAc (90 : 9 : 1, v/v/v) and centrifuged for 1 min at 9,600 g. Extraction of SA from the mixture was performed according to Segarra *et al.* (2006). Liquid chromatography-mass spectrometry (LC-MS) analyses were conducted using a UPLC-Triple Quadrupole-MS (Waters Xevo TQ, Milford, MS, USA). Samples were filtered through a Millex-HV Durapore (Bio-Rad laboratories, Hercules, CA, USA) [poly(vinylidene difluoride), PVDF] membrane (0.22 μm) before injection into the LC-MS instrument. Separation was performed on a 2.1 × 100 mm^2, 1.7-μm UPLC BEH C18 column. Chromatographic and MS parameters were as follows: the mobile phase consisted of water (phase A) and 0.1% formic acid in acetonitrile (phase B). The linear gradient program was as follows: 75% to 25% A over 0.1 min, 75% to 0% A over 5 min, held at 0% A over 1 min, and then returned to the initial conditions (75% A) over 1 min and held at 75% A for 6 min. The flow rate was 0.3 mL/min and the column temperature was kept at 35 °C. All the analyses were performed using the Electrospray ionization (ESI) source in negative ion mode with the following settings: capillary voltage, 3.2 kV; cone voltage, 30 V; desolvation temperature, 350 °C; desolvation gas flow, 650 L/h; source temperature, 150 °C. Quantification was performed using multiple reaction monitoring (MRM) acquisition by monitoring the 137/65 and 137/93 (Retention time (RT) = 2.05; dwell time of 61 ms for each transition) peaks for SA, and 141/97 (RT = 2.05; dwell time of 61 ms) peak for d4-SA (used as internal standard).

Statistical analysis

Results are expressed as means ± standard deviation. Statistical analysis was performed using JMP 5 software (SAS Institute Inc., 2002, Cary, NC, USA). Data were subjected to one-way analysis of variance (ANOVA) and Tukey honestly significant difference for comparison of means.

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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 *RDR1* gene expression in cucumber leaves.

Fig. S2 Expression of *CsRDR1c1* and *CsRDR1c2* was induced to similar levels by virus infection in cucumbers 'Shimshon' and 'Bet-Alfa'.

Fig. S3 Amino acid sequence alignment of putative proteins encoded by the *CsRDR1a*, *CsRDR1b*, *CsRDR1c1* and *CsRDR1c2* genes of cucumber 'Bet-Alfa' and the *AtRDR1a* gene of *Arabidopsis thaliana*.

Fig. S4 *RDR1* gene expression in cucumber roots.

Fig. S5 Time course analysis of *CsRDR1b* and *CsRDR1c1* expression following *Cucumber mosaic virus* (CMV) infection in cucumber.

Table S1 List of RNA-dependent RNA polymerase (RDR) accession numbers.

Table S2 List of viruses tested.

Table S3 Primers used in this study.