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Involvement of *RDR6* in short-range intercellular RNA silencing in *Nicotiana benthamiana*

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In plants, non-cell autonomous RNA silencing spreads between cells and over long distances. Recent work has revealed insight on the genetic and molecular components essential for cell-to-cell movement of RNA silencing in *Arabidopsis*. Using a local RNA silencing assay, we report on a distinct mechanism that may govern the short-range (6–10 cell) trafficking of virus-induced RNA silencing from epidermal to neighbouring palisade and spongy parenchyma cells in *Nicotiana benthamiana*. This process involves a previously unrecognised function of the RNA-dependent RNA polymerase 6 (*RDR6*) gene. Our data suggest that plants may have evolved distinct genetic controls in intercellular RNA silencing among different types of cells.

RNA silencing is a potent surveillance system to protect against virus infections in plants, fungi and animals. Viruses have evolved both passive and active strategies to escape and effectively suppress RNA silencing. In particular, viruses encode suppressors of RNA silencing (VSRs) that can directly or indirectly target various steps in intra-, intercellular and systemic RNA silencing[1]. Indeed, VSRs can subvert antiviral silencing by inhibiting the biosynthesis of small interfering (si)RNAs, by preventing the assembly of RNA-induced silencing complexes (RISC), by repressing the activity of the Argonaute (AGO) proteins, by impeding silencing amplification, and/or by blocking the spread of silencing signals. For example, the P38 coat protein (CP) of *Turnip crinkle virus* (TCV) is an effective VSR[2,3]. It binds to dsRNA and siRNA and specifically inhibits DCL4 activity to prevent siRNA production. TCV P38 also interacts with (and subsequently inactivates) AGO1 to impede RISC-mediated targeting and the degradation of viral RNAs[4–6]. The silencing suppression function of TCV P38 also requires the ethylene-inducible host transcription factor RAV2a[7].

In plants, RNA silencing is non-cell autonomous[8]. The 21-nt siRNA produced by DCL4 from inverted-repeat transgenes, acts as the primary silencing signal and spreads short-range over 10 – 15 cells. This type of intercellular trafficking of RNA silencing does not require RNA-dependent RNA polymerase 6 (RDR6). However, DCL4 also generates an RDR6-dependent 21-nt secondary siRNA for long-range (i.e., beyond 10 – 15 cells) signalling in systemic RNA silencing[9,10]. Genetic analysis in *Arabidopsis* has revealed three recessive *silencing-movement-deficient* (*smd1-3*) mutations which are compromised in short-range cell-to-cell movement of RNA silencing[9]. *SMD1* and *SMD2* are allelic to *RDR2* and to *NRPD1a*, respectively. Both genes are required for intercellular but not intracellular RNA silencing[11]. More recently, through cell-specific rescue of the DCL4 function and cell-specific suppression of the movement of RNA silencing, it has been shown that 21-nt siRNA duplexes probably represent a component of the mobile silencing signal that spreads between plant cells[12]. Interestingly, mobile silencing signals also include a range of small RNA (sRNA) species. Indeed, transgene-derived and endogenous 22-, 23- and 24-nt siRNAs are able to move between plant cells through plasmodesmata and traffic long distances via phloem transportation[13]. These findings have led to an elegant model that explains the genetic and molecular basis for limited (10 – 15 cell) spread of RNA silencing from companion cells to neighbouring parenchyma and mesophyll cells outside the vasculature in *Arabidopsis*[8–12]. It should be noted that in this model RNA silencing is triggered by

a long dsRNA-producing transgene or a synthetic 21-nt siRNA. Interestingly, plants have also been shown to be capable of hijacking viral RNA movement proteins in order to facilitate trafficking of virus-induced and transgene-mediated RNA silencing[14,15].

On the other hand, RNA silencing can be initiated by transgenes, high molecular weight RNAs, siRNA or plant RNA and DNA viruses in various cell types. In these scenarios, the initiation of silencing and the subsequent movement of silencing signals between cells may have different requirements. Using a local RNA silencing assay coupled with molecular and cellular analyses, we have uncovered a mechanism associated with short-range (6 – 10 cell) spread of virus-induced RNA silencing from individual epidermal cells to adjacent cells. This process requires a functional *RDR6* gene in *Nicotiana benthamiana*.

Results

RDR6 **is essential for cell-to-cell trafficking of local virus induced RNA silencing.** We exploited the *Turnip crinkle virus* (TCV)-based local RNA silencing assay devised to dissect the cell-to-cell spread of antiviral RNA silencing in plants[3,14]. The movement-deficient TCV-GFPΔCP was constructed by replacing the P38 CP gene with the green fluorescent protein (GFP) coding sequence[3]. Thus, it lacks the potent silencing suppressor CP and is restricted to single epidermal cells of *N. benthamiana*[2–5,7,12]. When mechanically applied to the upper epidermis of young leaves of GFP-expressing *N. benthamiana* GFP16c transgenic plants[16], TCV-GFPΔCP was able to initiate *gfp* RNA silencing in single epidermal cells. Then, silencing spread from cell-to-cell to form *gfp*-silenced foci, that were visible under long-wavelength UV light (Fig. 1a, b, e, f, i, j, m, n, q, r). Efficient and multi-dimensional spread of *gfp* silencing from a single epidermal cell to other cell types was evident (Table 1; Fig. 2a). Large numbers of silencing foci per inoculated leaf were counted on both the upper (161 ± 6) and lower (146 ± 12) epidermises of four leaves. Silencing foci, selected at random, appeared to have similar sizes (0.98 ± 0.39 mm, 0.97 ± 0.27 mm; n = 12) on upper and lower epidermis, respectively. Interestingly, we

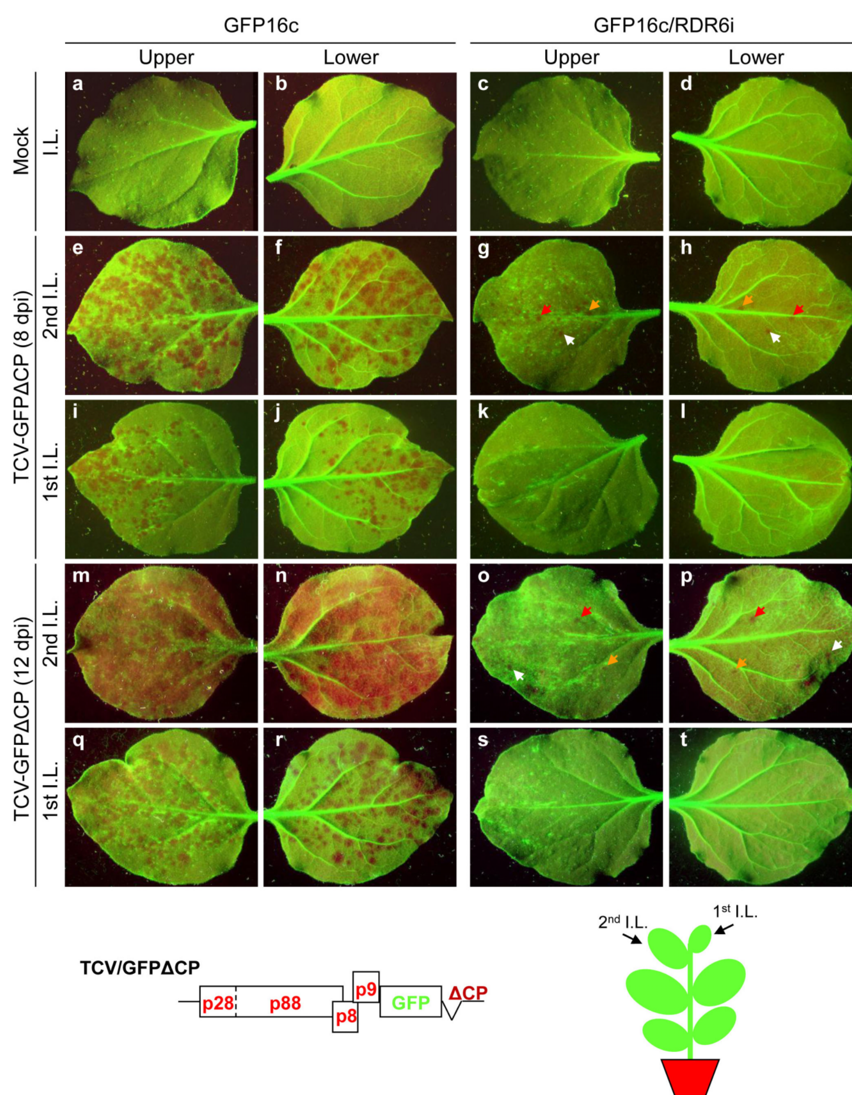

Figure 1 | Local induction and cell-to-cell spread of virus-induced RNA silencing. The first and second young leaves of *N. benthamiana* GFP16c and GFP16c/RDR6i at the six-leaf stage were mechanically inoculated with an equal amount of TCV-GFPΔCP RNA transcripts. Control plants were mock-inoculated with 10 mM Tris-HCl (pH 8.0) containing 10 mM EDTA. Both the upper and lower epidermises of inoculated leaves were photographed 8 days post-inoculation (dpi) (e–l) or 12 dpi (a–d, m–t) using a Nikon Coolpix995 digital camera under long-wavelength UV illumination through a yellow Kodak No. 58 filter. *Gfp* RNA-silenced tissue (foci) showed red chlorophyll fluorescence and *gfp*-expressing tissue showed green fluorescence. Colour-coded arrows in Panels (g), (h), (o) and (p) show the same *gfp* silencing foci observed from the upper or lower epidermis. A diagram showing the genome of TCV-GFPΔCP is included. A cartoon of a plant shows the positions of the first and second inoculated leaves (I. L.).

Table 1 | Impact of *RDR6* on cell-to-cell spread of virus-induced RNA silencing

		GFP16c	GFP16c/RDR6i	Student's *t*-test[3]
Numbers of silencing foci per leaf[1]	Upper	161 ± 6 (n = 4)	25 ± 25 (n = 4)	p = 0.00001**
	Lower	146 ± 12 (n = 4)	4 ± 4 (n = 4)	p = 0.00001**
	t-test[3]	p = 0.124*	p = 0.209*	
Diameters of silencing foci (mm)[2]	Upper	0.98 ± 0.39 (n = 12)	0.50 ± 0.35 (n = 12)	p = 0.002**
	Lower	0.97 ± 0.27 (n = 12)	0.51 ± 0.39 (n = 12)	p = 0.003**
	t-test[3]	p = 0.952*	p = 0.953*	

[1]Number of silencing foci were counted on the upper or lower epidermis of four leaves, one leaf from each of four inoculated plants.
[2]Diameters of 12 randomly selected silencing foci were measured using a micro-ruler under long-wavelength UV light.
[3]Student's *t*-tests were carried out between the upper and lower epidermis*. The *t*-tests were also performed between GFP16c and GFP16c/RDR6i**. There are significant differences in the average numbers and sizes of silencing foci between GFP16c and GFP16c/RDR6i, but not between the upper and lower epidermis of plants with a same genetic background.

noticed that the number of silencing foci was always higher in the 2nd inoculated leaf than in the 1st inoculated leaf (Fig. 1), suggesting that the age of leaf tissues might have an effect of local RNA silencing. These data were consistent with previous results[3,14,17]. However, in GFP16c/RDR6i plants in which *RDR6* was silenced[16], we found a marked reduction in the numbers of visible *gfp* silencing foci, on the upper (25 ± 25) and, particularly the lower (4 ± 4) epidermis of four treated leaves (Table 1; Fig. 1c, d, g, h, k, l, o, p, s, t; Fig. 2a). There was also a significant decrease in the average size (diameter) of the *gfp* silencing foci on the upper (0.50 ± 0.35 mm) and lower (0.51 ± 0.39 mm) epidermises (Table 1; Fig. 2b). This phenomenon was observed in all repeated (more than three separate) experiments. Thus, in *RDR6*-"knockdown" plants, the silencing initiated by TCV-GFPΔCP in individual upper epidermal cell was incompetent to spread between epidermal cells and to penetrate to palisade and spongy parenchyma cells in order to reach lower epidermis. These findings suggest that *RDR6* was required for the cell-to-cell communication of RNA silencing in *N. benthamiana*. It should be noted that sizes of individual silencing foci could vary because the initiation of intracellular silencing may not be synchronous and the development of each silencing focus is probably a dynamic process.

***RDR6* is required for the short-range (6 – 10 cell) intercellular spread of virus-induced RNA silencing.** We examined 12 randomly selected silencing foci by fluorescent microscopy and estimated the numbers of epidermal cells in which *gfp* RNA silencing had occurred (Fig. 2c–l). A typical silencing focus that formed in GFP16c leaves inoculated with TCV-GFPΔCP consisted of 100–300 epidermal cells, equivalent to a circular zone with a radius of 6 – 10 epidermal cells (Fig. 2c, d, g, h, k). These data demonstrate that virus-induced RNA silencing can move from a single upper epidermal cell over a short-range of 6 – 10 upper epidermal cells. However, intracellular RNA silencing originating from a single upper epidermal cell could move, simultaneously, in three-dimensions to and through many more cell types, including palisade, mesophyll and lower epidermal cells, to form a visible focus of silencing (Fig. 1). Such RNA silencing was unlikely to move into the vasculatures as systemic RNA silencing was not observed in distal leaf tissues[3,14,17]. Consistent with their reduced size (Fig. 2b), the *gfp* RNA silencing foci that formed on GFP16c/RDR6i leaves encompassed only 30 – 100 epidermal cells, equivalent to a circular zone with a radius of 3–6 epidermal cells (Fig. 2e, f, i, j, l). These data provide additional evidence that a functional *RDR6* gene plays an important role in short-range (6 – 10 cell) trafficking of virus-induced RNA silencing.

***RDR6* does not influence the replication and defective movement of TCV-GFPΔCP in *N. benthamiana*.** To address whether *RDR6* gene expression could affect replication and cell-to-cell spread of TCV-GFPΔCP, we first performed quantitative RT-PCR (qRT-PCR) assays to detect and compare the levels of *RDR6* mRNA in wild-type and *RDR6*-silenced lines NbRDR6i and GFP16c/RDR6i[16] using two housekeeping gene *GAPDH* and *EF1α* transcripts as internal controls. Our data showed that *RDR6* gene expression was statistically significantly reduced in NbRDR6i and GFP16c/RDR6i plants when compared to that in the wild-type *RDR6* (Nb and GFP16c) plants (Fig. 3a, b; Table S1). Moreover, qRT-PCR analyses showed that TCV-GFPΔCP RNA accumulation reached equivalent levels with no statistical significance in wild type *N. benthamiana* (Nb) and *RDR6*-silenced NbRDR6i (Fig. 3a, b; Table S1). We also showed that the levels of TCV-GFPΔCP RNA in the inoculated leaves of GFP16c and GFP16c/RDR6i were similar, but decreased substantially when compared to the viral RNA levels in wild-type Nb or *RDR6*-silenced NbRDR6i plants, respectively (Fig. 3a, b; Table S1). This is suggesting a partial resistance to TCV-GFPΔCP in the GFP16c lines. Such resistance may be derived from homologous sequence-dependent specific targeting to the GFP marker gene, which then resulted in degradation of the recombinant virus by RNA silencing. It is worthwhile noting that the transgenic GFP16c line and the wild-type *N. benthamiana* plants are susceptible to infection with TCV, *Tobacco mosaic virus* and *Potato virus X*[3,17,18], suggesting the GFP16c line should not have abnormalities in viral immunity at least for these viruses. Furthermore, TCV-GFPΔCP was always found to be restricted to single epidermal cells with a wild-type or *RDR6*-silenced genetic background (Fig. 3c, d). These data indicate that TCV-GFPΔCP was an effective inducer and the target of intracellular RNA silencing, and that *RDR6* was not required for the induction of antiviral RNA silencing in individual epidermal cells.

Taken together, our findings support the hypothesis that *RDR6* is probably involved in promoting the cell-to-cell spread of TCV-GFPΔCP induced RNA silencing, and the significant decrease in the intercellular RNA silencing in GFP16c/RDR6i plants is not due to any impact from *RDR6* on viral RNA replication and intercellular virus trafficking.

Discussion

Plants have multiple *RDR* genes that function in different biological processes. In *Arabidopsis*, there are six *RDR* genes (*RDR1-6*) that encode potential RNA dependent RNA polymerases (RDRs), of which *RDR6* is the major *RDR* acting in post-transcriptional gene silencing and virus induced RNA silencing[11,19,20], while *RDR2* plays an important role in transcriptional gene silencing[21]. *RDR6*-dependent RNA silencing forms an important antiviral defence. It defends both differentiated and meristematic tissues from viral invasion. *RDR6* silencing causes plants to be hyper-susceptible to virus infection[16,22,23]. *RDR1* is also involved in antiviral RNA silencing, but its effects on plant responses to viral invasion tend to be virus specific[24–27]. Recently, small RNA deep sequencing revealed a role for *RDR1* and *RDR6* in the biogenesis of viral siRNA[24,28] and showed *RDR6* was essential for the maintenance and transitivity of RNA silencing[19,29–31]. In *Arabidopsis*, *RDR6* is also thought to play a role in long-range

Figure 2 | Influence of *RDR6* on intercellular RNA silencing. (a–b) The *RDR6* gene affects the intercellular spread of TCV-GFPΔCP-induced *gfp* silencing. The average numbers of silencing foci per inoculated leaf for four leaves in a typical experiment using recombinant RNA transcripts produced from 2.5 μg of TCV-GFPΔCP DNA template are shown (a). Numbers of foci were counted 8 days post-inoculation (dpi). The average sizes of 12 randomly selected silencing foci from the upper and lower epidermises were analysed (b). (c–l) Dark *gfp* RNA silencing foci from the upper and lower epidermises of *N. benthamiana* GFP16c and GFP16c/RDR6i were examined under a fluorescence microscope using a green filter (c–f) or through bright field illumination (g–l). The boxed areas were used to estimate the numbers of epidermal cells in which *gfp*-silencing occurred. Individual epidermal cells are outlined and marked with asterisks. Scale bars are indicated.

(> 10 – 15 cell) intercellular and systemic RNA silencing, but not in short-range (10 – 15 cell) cell-to-cell signalling of RNA silencing that was initiated in companion and phloem cells[10,16,32].

However, considering the fact that TCV-GFPΔCP is restricted to single epidermal cell of mechanically inoculated leaves, our data suggest that the short-range (6 – 10 cell) intercellular trafficking of virus-induced RNA silencing from a single epidermal cell to adjacent palisade and spongy parenchyma cells in *N. benthamiana* may require a functional *RDR6* gene (Table 1; Fig. 1; Fig. 2). It is possible that spread of this type of RNA silencing may be associated with a small RNA signal[8]. The RDR6 protein is known to bind ssRNA, dsRNA and even dsDNA in plants[33]. Thus, RDR6 and small RNAs could form an RNA-protein complex that may directly promote 6 – 10 cell-to-cell movement of silencing signal. This model is consistent with the localization of the RDR6 protein in the cytoplasm, the nucleus and the cell membrane[34,35]. Alternatively, RDR6 may be required to produce a signal for limited intercellular silencing. Single cells with a normal RDR6 function in GFP16c plants could generate sufficient quantities of mobile signal molecules to enable the formation of silencing foci. In contrast, reduced RDR6 activity in

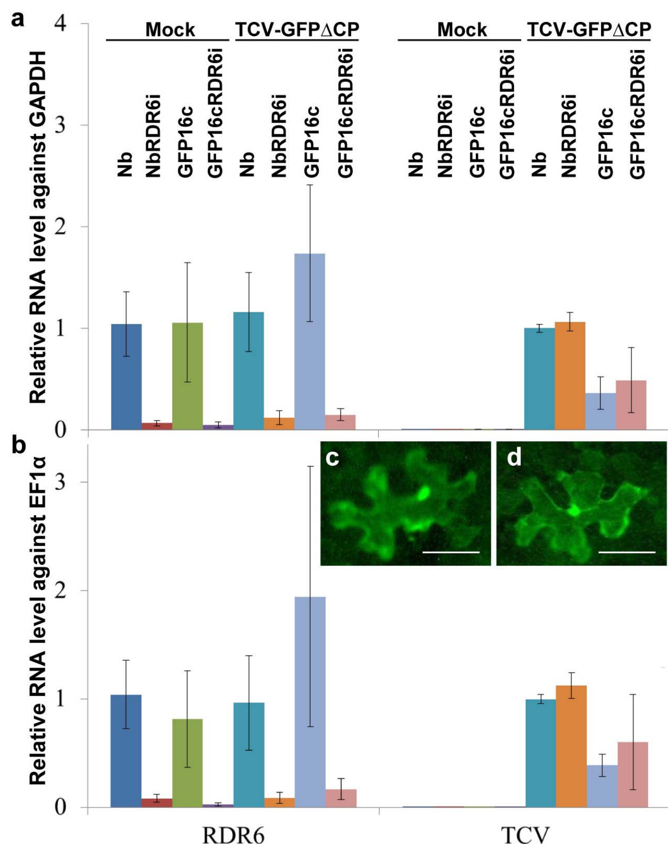

Figure 3 | ***RDR6*-independent intracellular RNA silencing.** (a and b) The *RDR6* gene does not affect replication of TCV-GFPΔCP. The *RDR6* mRNA were analysed 12 days post-inoculation (dpi) by quantitative (q) RT-PCR, in triplicate, using total RNAs extracted from mock- or TCV-GFPΔCP-inoculated leaves of wild-type *N. benthamiana* (Nb) and NbRDR6i (RDR6i), transgenic GFP 16c (GFP16c) and GFP16c/RDR6i plants. Virus-induced intracellular RNA silencing targets TCV-GFPΔCP. *TCV-GFP* RNA was analysed by qRT-PCR in triplicate, using total RNAs extracted from mock- or TCV-GFPΔCP-inoculated GFP16c and GFP16c/RDR6i leaves at 12 dpi. *N. benthamiana* housekeeping *GAPDH* (a) and *EF1α* (b) transcripts were used as internal controls. Relative RNA levels of *RDR6* or TCV were obtained by normalising against the baseline expression levels of *GAPDH* (a) and *EF1α* (b) mRNA, respectively, and showed similar tendencies between the two internal controls. Student's *t*-tests were carried out to evaluate whether there would be any statistical significance in RNA levels between different biological samples (Table S1). (c and d) Fluorescence microscopic examination of *gfp* expression in a single epidermal cell in TCV-GFPΔCP-inoculated Nb (c) or NbRDR6i (d) leaves 6 dpi using a Zeiss Axiophot microscope through a green filter. Bar = 100 μm.

GFP16c/RDR6i plants could lead to decreased synthesis of such a signal. Thus, the level of the mobile signal may be an important factor for intercellular RNA silencing. In this scenario, RDR6 may play an indirect role in short-range (6 – 10 cell) trafficking of RNA silencing. Also supporting a basic role of RDR6, not only the size but also the number of foci is lower in the RDR6-deficient plants. Moreover, some silencing foci were detected in the lower epidermis of leaves inoculated in their upper faces (Fig. 1). However, this could be caused by that *RDR6* was not completely knocked out and residual RDR6 activity might persist in the RDR6i plants. Indeed, low but quantifiable levels of *RDR6* gene expression were readily detectable in all *RDR6*-silenced plants (Fig. 3a, b). A third possibility could be that indirect impacts of the knockdown of *RDR6* on the plant defensive responses, or even on the physiological transport of macro-molecules might affect the cell-to-cell spread of virus induced RNA silencing. It should be noted that the cell-to-cell spread of RNA silencing in TCV-GFPΔCP/GFP16c-based local RNA silencing assay depends on the expression of functional P8 and P9 proteins of TCV[14]. This assay is also susceptible to different VSRs[17]. These factors may affect how RDR6 protein is recruited to promote 6 – 10 cell-to-cell spread of virus-induced RNA silencing, a process that may have different genetic and molecular requirements in *Arabidopsis* and other plants[8-12]. Regardless of the underpinning mechanism, our data have revealed a previously unknown function for the *RDR6* gene in short-range (6 – 10 cell) intercellular RNA silencing in *N. benthamiana*.

Our unexpected finding contradicts previous reports that RDR6 was not involved in the limited 10 – 15 cell-to-cell spread of RNA silencing in *Arabidopsis*[8-12]. Such a discrepancy may be due to a combination of factors (Table S2). Compared with *Arabidopsis*, *N. benthamiana* is permissive and can be a non-native host for a wide range of plant viruses, supposedly due to a naturally occurring mutation in the *N. bethamiana RDR1* gene[26,27]. Indeed, *N. benthamiana* and *Arabidopsis* plants respond differently to TCV-GFPΔCP. For instance, TCV CP is essential for cell-to-cell virus movement in *N. benthamiana*[3,36], but not in *Arabidopsis*[5]. Consequently, TCV-GFPΔCP moved effectively from cell-to-cell to form multi-cellular lesions in inoculated *Arabidopsis* leaves (unpublished data). However, the same virus was restricted to single cell in wild-type and RDR6i *N. benthamiana* plants (Fig. 3c, d). Such distinctive virus-host responses and different genetic backgrounds may affect the biosynthesis and spread of the silencing signal, thus have a significant impact on TCV-GFPΔCP-mediated local RNA silencing in these plant species.

On the other hand, confinement of virus infection to individual epidermis cells was only demonstrated in plants not expressing GFP (Fig. 3c, d), but not directly in the centre of the silenced foci of the GFP16c and GFP16c/RDR6i plants or in single infected cells that are not surrounded of silenced foci in the GFP16c/RDR6i plants. However, we had checked hundreds of silencing foci under epifluorescent and confocal microscopes, no individual epidermal cells with viral transient GFP fluorescence in the centre of silencing foci were ever found, although GFP-expressing cells of Nb and NbRDR6i leaves inoculated with TCV-GFPΔCP were readily observed at 2 – 12 dpi. Virus can be a trigger and a target of virus-induced RNA silencing (Fig. 3a, b). Once a silencing focus became visible, virus-induced RNA silencing would be certainly well-established and it could target viral RNAs for degradation. Therefore, accumulation of viral RNAs and proteins in single epidermal cells could be reduced to an undetectable level. Nevertheless, our findings emphasise that the signalling of intercellular RNA silencing is a more complex process than previously proposed (Table S2). This process may have different molecular components in different cell types in same plants and exhibit distinct genetic requirements in different plant species.

Methods

Plasmid construction, inoculation and plant maintenance. Plasmid TCV-GFPΔCP has been described[3]. Wild-type (Nb) and *RDR6*-knocked down (NbRDR6i)[16] *N. benthamiana* and *Arabidopsis* plants were inoculated with TCV-GFPΔCP as described[3,37]. Plants were maintained in an insect-free glasshouse at 25°C with supplementary lighting to give a 16-hour photoperiod. To measure the accumulation of viral RNA, total RNAs were extracted from tissues using the RNeasy Plant Minikit (Qiagen) as described[17]and assayed by Quantitative RT–PCR (qRT-PCR).

Local RNA silencing assay. Two-to-four seedlings of *gfp*-expressing transgenic lines of *N. benthamiana* (GFP16c and GFP16c/RDR6i)[16] were mechanically inoculated with RNA produced by *in vitro* transcription from linearised TCV-GFPΔCP plasmid DNA in three separate experiments. The induction of intercellular *gfp* RNA silencing was examined under long-wavelength UV light and recorded photographically through a Kodak No. 58 filter using a Nikon Digital Camera Coolpix995 as described[14]. Regions of leaf lamina in which silencing of *gfp* RNA occurred show only red chlorophyll fluorescence, while tissues expressing GFP show green fluorescence.

qRT-PCR assay. Virus or mock-inoculated leaves of 3 plants (Nb, NbRDR6i, GFP16c and GFP16c/RDR6i) were separately taken at 12 days post inoculation in repeated

experiments for RNA extraction[17]. The first-stranded cDNA was synthesized using total RNAs treated with RNase-free DNase I as templates by the M-MLV Reverse Transcriptase (Promega). The qRT–PCR analyses of viral and *RDR6* mRNA levels were performed using specific primers (Table S3) and the iQ™ SYBR Green Supermix. The amplification program for SYBR Green I was performed at 95°C for 10 seconds and 55°C for 30 seconds on the CFX96 machine (Bio-Rad), following the manufacturer's instructions. Triplicate quantitative assays were performed on cDNA of each biological sample. The relative quantification of viral or *RDR6* mRNA was calculated using the formula $2^{-\triangle\triangle Ct}$ and normalized to the amount of *GAPDH* (Genbank accession number TC17509) or *EF1α* (Genbank accession number AY206006) mRNA detected in the same sample, respectively.

Fluorescence and laser confocal microscopy. Mock- or virus-inoculated leaves were collected, examined under a Zeiss Axiphot microscope through a green filter and photographed with a Nikon Coolpix995 digital camera[38,39], or under an LSM 710 Laser Scanning Microscope using settings to visualise GFP green and chlorophyll red fluorescence[40].

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Author contributions

C.Q., N.S., M.G., H.Z., B.L., J.S. and A.M. designed and performed experiments, C.L. and E.R. performed researches, H.W., Y.L., T.O. and M.V. contributed through discussions and revised the paper. Y.H. initiated the project, designed the experiments and wrote the paper.

Additional information

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