Roles of Dicer-Like Proteins 2 and 4 in Intra- and Intercellular Antiviral Silencing

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RNA silencing is an innate antiviral mechanism conserved in organisms across kingdoms. Such a cellular defense involves DICER or DICER-LIKEs (DCLs) that process plant virus RNAs into viral small interfering RNAs (vsiRNAs). Plants encode four DCLs that play diverse roles in cell-autonomous intracellular virus-induced RNA silencing (known as VIGS) against viral invasion. VIGS can spread between cells. However, the genetic basis and involvement of vsiRNAs in non-cell-autonomous intercellular VIGS remains poorly understood. Using GFP as a reporter gene together with a suite of RNAi transgenic lines, we show that despite the well-established activities of DCLs in intracellular VIGS and vsiRNA biogenesis, DCL4 acts to inhibit intercellular VIGS whereas DCL2 is required (likely along with DCL2-processed/dependent vsiRNAs and their precursor RNAs) for efficient intercellular VIGS trafficking from epidermal to adjacent cells. DCL4 imposed an epistatic effect on DCL2 to impede cell-to-cell spread of VIGS. Our results reveal previously unknown functions for DCL2 and DCL4 that may form a dual defensive frontline for intra- and intercellular silencing to double-protect cells from virus infection in Nicotiana benthamiana.

RNA silencing targets endogenous cellular nucleic acids and exogenous invasive pathogenic RNAs or DNAs for homologous RNA-dependent degradation, translation repression, or RNA-directed DNA methylation (RdDM) in eukaryotic organisms (Baulcombe, 2004; Sarkies and Miska, 2014). In plants, RNA silencing forms an innate defense against virus infection (Aliyari and Ding, 2009; Csorba et al., 2015). Such an antiviral mechanism involves DICER-LIKE (DCL) RNase type III enzymes. Most plants encode four DCLs of which DCL1 is responsible for production of microRNA, whereas DCL2, DCL3, and DCL4 are responsible for biogenesis of 22-, 24-, and 21-nucleotide small interfering RNA (siRNA), respectively (Mukherjee et al., 2013). DCL2 and DCL4 possess partially redundant functions in the production of transacting siRNA, but DCL2 acts predominantly to manufacture various-sized secondary siRNAs (Chen et al., 2010; Henderson et al., 2006; Xie et al., 2005). Unlike animal viruses, plant viruses have not yet been found to encode any microRNA or specific site that can be targeted by host cellular microRNAs. However, artificial microRNAs can inhibit plant virus infection (Qu et al., 2007). In Arabidopsis (Arabidopsis thaliana), DCLs can process plant virus RNAs into viral small interfering RNAs (vsiRNAs) within individual cells. For instance, DCL4 and DCL4-processed 21-nucleotide vsiRNAs are involved in virus-induced RNA silencing (also known as VIGS), a kind of posttranscriptional gene silencing (PTGS; Bouché et al., 2006; García-Ruiz et al., 2010; Qu et al., 2008). DCL2 and its cognate 22-nucleotide vsiRNAs may also affect VIGS in plant cells when DCL4 is absent or defective (Andika et al., 2015; Wang et al., 2011; Zhang et al., 2012). On the other hand, DCL3 and 24-nucleotide vsiRNAs are associated with RdDM and transcriptional gene silencing (TGS) in the protection of plant cells from DNA virus infection (Aregger et al., 2012; Blevins et al., 2006). In Arabidopsis, DCL4 and DCL2 also play hierarchical and redundant roles in intracellular antiviral silencing (Bouché et al., 2006; García-Ruiz et al., 2010; Wang et al., 2011). Recently, a combined activity of DCL2 and DCL3 has been reported to be critical in defending plants from viroid infection (Katsarou et al., 2016). DCL1 can negatively regulate the DCL4-initiated antiviral RNA silencing pathway (Qu et al., 2008). However, the roles of the different DCLs in promoting intercellular VIGS for plant systemic acquired resistance to virus infection are unclear. In response to virus infection, intracellular VIGS in the initial virus-infected cells triggers intercellular...
silencing in adjacent cells, which spreads systemically to remote tissues. This is known as non-cell-autonomous VIGS. Non-cell-autonomous VIGS combats incoming viruses and protects recipient cells from further viral invasion (Schwach et al., 2005). In Arabidopsis, spread of the phloem-originating PTGS from companion cells to nearby cells requires DCL4 and DCL4-processed 21-nucleotide siRNA signals (Dunoyer et al., 2005). However, whether 21-nucleotide siRNAs represent the bona fide silencing signals that are transportable among plant cells is highly controversial (Berg, 2016). On the other hand, DCL2 can stimulate transitive PTGS and biogenesis of secondary siRNAs (Mlotshwa et al., 2008). DCL2 can also restore silencing in the Arabidopsis dcl4 mutant that is deficient in cell-to-cell spread of transgene-mediated PTGS (Parent et al., 2015). Moreover, intercellular and systemic PTGS involve many cellular factors including RDR6, which has been shown to be required for efficient cell-to-cell movement of VIGS (Melnik et al., 2011; Qin et al., 2012; Searle et al., 2010; Smith et al., 2007). Nonetheless, the genetic basis and the requirement of vsiRNAs for cell-to-cell and systemic spread of antiviral VIGS remain to be elucidated.

We previously developed a Turnip Crinkle Virus (TCV)-based local silencing assay to investigate intra- and intercellular VIGS in Nicotiana benthamiana (Qin et al., 2012; Ryabov et al., 2004; Shi et al., 2009; Zhou et al., 2008). TCV belongs to Carmovirus with a single positive-stranded RNA genome (Carrington et al., 1989). It encodes five proteins, namely, the RNA-dependent RNA polymerases P28 and its read-through P88; movement proteins P8 and P9; and coat protein (CP) P38 (Carrington et al., 1989; Hacker et al., 1992; Li et al., 1998).

RESULTS

DCL RNAi Does Not Affect Cell-to-Cell Mobility of TCV/GFPΔCP

To dissect the genetic requirements and silencing signals involved in non-cell-autonomous intercellular VIGS (Fig. 1) in N. benthamiana (Nb), we utilized a suite of DCL RNAi transgenic Nb lines including DCL1i; DCL2Ai and DCL2Bi; DCL3Ai and DCL3Bi; DCL4Ai and DCL4Bi; and one double RNAi line DCL2i DCL4i (Supplemental Table S1). We also used GFP transgenic lines 16cGFP; GfpDCL1i; and lines Gfp; GfpDCL2Ai and GfpDCL2Bi; GfpDCL3Ai and GfpDCL3Bi; GfpDCL4Ai and GfpDCL4Bi, which were derived from crosses between 16cGFP and Nb or DCL RNAi lines, respectively, as well as a triple cross line GfpDCL2i DCL4i (Supplemental Table S1). We performed qRT-PCR assays and revealed that DCL2, DCL3, and DCL4 transcript levels were down-regulated by 60 to 80% in each of the two independent RNAi lines; but only ~40% reduction was achieved for DCL1i in DCL1i (Fig. 1A). We then analyzed the impact of DCL RNAi on cell-to-cell mobility of TCV/GFPΔCP (Fig. 1, B–G). The upper epidermis of leaves in each DCL RNAi plant at the six-leaf stage were inoculated with TCV/GFPΔCP. As observed under the fluorescent microscope, strong GFP green fluorescence appeared only in single epidermal cells in leaves of the wild-type Nb control (Fig. 1C) and all DCL RNAi plants (Fig. 1, D–G). These results demonstrate that presence of TCV/GFPΔCP was limited to individual virus-infected cellular and that DCL RNAi did not affect the movement-deficiency of TCV/GFPΔCP.
DCL4 RNAi Enhances, Whereas DCL2 RNAi Reduces, Cell-to-Cell Spread of VIGS

To test whether intra- and intercellular VIGS is affected by the down-regulation of individual DCL genes, we used GFP as a reporter and mechanically inoculated the movement-deficient TCV/GFPΔCP onto young leaves of 16cGFP (Fig. 1H), GfpDCL1i (Fig. 1I), Gfp (Fig. 1J), GfpDCL2Ai and GfpDCL2Bi (Fig. 1, K and L), GfpDCL3Ai and GfpDCL3Bi (Fig. 1, M and N), and GfpDCL4Ai and GfpDCL4Bi (Fig. 1, O and P) plants. We then counted the number of GFP silencing foci on both upper and lower epidermises of the inoculated leaves, and measured sizes in diameter of 80 to 560 randomly selected silencing foci on the upper epidermis. Student’s t tests were performed for qRT-PCR and silencing data (mean ± se) and P values are indicated (asterisks).
Supplemental Table S2). DCL2 RNAi also caused a reduction in the number of silencing foci per leaf (Fig. 1R; Supplemental Table S2). RNAi knockdown of DCL1 or DCL3 did not affect the number of silencing foci and only reduced cell-to-cell movement of VIGS to a small extent, as evidenced by 3 to 5% decreases in silencing foci sizes and/or some reductions in SCI (Fig. 1, H–J, M, N, and R–U; Supplemental Table S2). This suggests that DCL3 and/or DCL1 may not contribute significantly to intercellular VIGS. However, a possible role cannot be ruled out completely due to discrepancy between the two GfpDCL3i lines and because we only have data from a single GfpDCL1i line. As with DCL2 RNAi, DCL4 RNAi caused a reduction in the number of silencing foci in GfpDCL4Ai and GfpDCL4Bi plants (Fig. 1R; Supplemental Table S2). This is consistent with the predominant role that DCL4 plays in intracellular VIGS. To our surprise, the average sizes of silencing foci increased by ≥20% (Fig. 1, O, P, S, and T; Supplemental Table S2). The SCI also raised from ~58% in the Gfp controls to 70 to 75% in the two GfpDCL4 RNAi lines (Fig. 1U; Supplemental Table S2). These results demonstrate that DCL4 RNAi reduced intracellular silencing, but enhanced intercellular spread of VIGS. Taken together, our findings show that DCL4 RNAi enhances but DCL2 RNAi reduces cell-to-cell spread of VIGS in Nb.

**DCL4 Interferes with DCL2 to Control Intercellular VIGS**

To investigate whether DCL4 and DCL2 would affect each other to influence cell-to-cell spread of VIGS in Nb, we inoculated the triple-cross GfpDCL24i plant with TCV/GFPACP. We found a marked reduction in the number of GFP silencing foci (Fig. 1R; Supplemental Table S2), consistent with the reduction in the number of silencing foci observed in GfpDCL2 RNAi and GfpDCL4 RNAi lines. However, in the triple-cross plants, the average sizes of silencing foci decreased by more than 40%, and SCI also fell from 58 to 44% when compared to the Gfp control (Fig. 1, Q, S, and T; Supplemental Table S2). These results demonstrate that simultaneous RNAi of DCL2 and DCL4 reduced both intra- and intercellular viRNAs, similar to what is seen in GfpDCL2 RNAi lines, but to a greater extent. The inhibition of intercellular spread of VIGS in the triple-cross GfpDCL24i line is opposite to the increase in intercellular VIGS seen in the GfpDCL4 RNAi lines. This implies that DCL4 imposed an epistatic effect on DCL2 to affect intercellular VIGS. This conclusion is supported by data from qRT-PCR assays (Fig. 2). DCL2 RNAi had no obvious impact on mRNA levels of DCL1, DCL3, and DCL4 (Fig. 2A). However, DCL4 RNAi led to a 20 to 40% increase in DCL2 expression but had no substantial influence on the transcript levels of DCL1 or DCL3; DCL3 RNAi also enhanced the level of DCL2 transcripts, but did not affect expression of DCL1 or DCL4 (Fig. 2B and C). Together with the specific RNAi effects on each DCL (Supplemental Data Set S1), these data reveal that DCL4 is involved in a negative regulation of DCL2 expression and as a consequence affecting the intercellular spread of VIGS in Nb.

**DCLs Play Differential Roles in viRNA Biogenesis**

To further understand how DCLs contribute to intra- and intercellular VIGS, we performed next-generation sequencing of sRNA libraries for mock- or TCV/GFPACP-inoculated Gfp, GfpDCL1i, GfpDCL2Ai, GfpDCL3Bi, and GfpDCL4Ai (Supplemental Text S2; Supplemental Data Sets S1–S3; Supplemental Fig. S1). We then mapped vsiRNAs and TcvGFP siRNAs onto the sequence of TCV/GFPACP (Fig. 3; Supplemental Fig. S2) and TCVACP (Supplemental Figs. S3 and S4). We found abundant vsiRNAs in TCV/GFPACP-inoculated RNAi lines (Fig. 3, A–E; Supplemental Fig. S3, A–E), compared to their mock controls (Supplemental Figs. S2, A–E, and S4, A–E; Supplemental Table S3). This is consistent with induction of effective VIGS in these plants (Fig. 1, H–U; Supplemental Table S2). More vsiRNAs were recorded in GfpDCL1i, GfpDCL2Ai, and GfpDCL3Bi plants (Fig. 3, B–D; Supplemental Fig. S3, B–D) than in Gfp controls (Fig. 3A; Supplemental Fig. S3A; Supplemental Table S3). However, the reads of vsiRNAs, particularly in the sense polarity, decreased in GfpDCL4Ai plants (Fig. 3E; Supplemental Fig. S3E; Supplemental Table S3) despite a marked increase in the overall number of siRNAs (vsiRNAs and TcvGFP-siRNAs) mapped to TCV/GFPACP (Supplemental Table S3). These results are consistent with the reduced level of recombinant viral RNAs in TCV/GFPACP-inoculated DCL RNAi plants, compared to the non-RNAi controls (Supplemental Fig. S5). On the other hand, the distribution of vsiRNAs across TCV/GFPACP (Fig. 3, A–E) or TCVACP (Supplemental Fig. S3, A–E) was identical among all virus-inoculated RNAi and control plants. Taken together, these data demonstrate that DCL4 is able to efficiently target viral RNAs for the production of vsiRNAs during cell-autonomous VIGS. Our results also reveal that DCL2 is required for cell-to-cell spread of VIGS, and DCL2 could target viral RNA and TcvGFP mRNA for degradation in Nb.

**Antagonistic Influences of DCL4 and DCL2 on Accumulation of siRNAs Associated with Intercellular VIGS**

In contrast to the situation with vsiRNAs, GfpDCL RNAi lines differed in the generation of TcvGFP or transgene 16cGFP siRNAs (dubbed “siRNA_{TcvGFP}” and “siRNA_{16cGFP}”) that are associated with intra- and intercellular VIGS. Note that the 714-nucleotide TcvGFP (Ryabov et al., 2004) and 792-nucleotide 16cGFP (Haseloff et al., 1997; Ruiz et al., 1998) mRNAs are not identical. Sequences between nucleotides 237 to 306 and 345 to 540 in TcvGFP (designated TcvGFP_{237-306} and TcvGFP_{345-540}) differ from the corresponding regions 300 to 369 and 408 to 603 in 16cGFP (designated...
Compared to the Gfp control and GfpDCL1i and GfpDCL3Bi plants, the levels of siRNA TcvGFP and siRNA 16cGFP were reduced in GfpDCL2Ai, but significantly increased in GfpDCL4Ai (Supplemental Table S3). When mapped the siRNAs onto TcvGFP (Fig. 4; Supplemental Fig. S7) and 16cGFP mRNA (Fig. 5; Supplemental Fig. S8). The distribution of sense and antisense GFP siRNAs to regions that are identical in TcvGFP and 16cGFP was essentially the same in Gfp and in each of the DCL RNAi lines, but the levels of siRNA TcvGFP and siRNA 16cGFP were lower in GfpDCL2Ai, and much higher in GfpDCL4Ai, compared to Gfp, GfpDCL1i, and GfpDCL3Bi (Figs. 4, A–E, and 5, A–E; Supplemental Table S3). Moreover, in GfpDCL4Ai the level of siRNA 16cGFP (2.5 million reads) was approximately double compared to the abundance of siRNA TcvGFP (1.28 million reads; Supplemental Table S3). Such substantial differences between siRNA TcvGFP and siRNA 16cGFP levels suggest that the transgene 16cGFP mRNA was targeted and diced by intra- and intercellular VIGS to a greater extent than TcvGFP transcripts. In contrast, different profiles were observed for siRNA TcvGFP and siRNA 16cGFP corresponding to the two less-similar regions (Region 1: TcvGFP 237-306 and 16cGFP 300-369; Region 2: TcvGFP 345-540 and 16cGFP 408-603; Figs. 4, A–E, and 5, A–E).
However, higher levels of 16cGFP_{308-603} and 16cGFP_{408-603} siRNAs were observed, a significant amount of which was antisense. As with the other GFP siRNAs, the levels of 16cGFP_{308-603} and 16cGFP_{408-603} siRNAs were much higher in GfpDCL4Ai and lower in GfpDCL2Ai, compared to Gfp, GfpDCL1i, and GfpDCL3Bi (Fig. 5, A–E).

These results demonstrate that DCL4 and DCL2 antagonistically affected the accumulation of siRNAs associated with intercellular VIGS. The reduction of siRNA_{TcvGFP} and siRNA_{16cGFP} in GfpDCL2Ai or the massive accumulation of these siRNAs in GfpDCL4Ai is likely to be due to the respective loss- or gain-of-function of DCL2-dependent production of primary or secondary siRNAs in these RNAi lines.
Figure 4. Distribution of 20- to 25-nucleotide GFP siRNAs across the 714-nucleotide TcvGFP mRNA. A, Gfp. B, GfpDCL1i. C, GfpDCL2Ai. D, GfpDCL3Bi. E, GfpDCL4Ai. The sRNA libraries were generated from sRNA samples extracted from TCV/GFPΔCP-inoculated leaves. Blue and red bars represent siRNAs aligned to the sense (positive) and antisense (negative) strands of TcvGFP mRNA, respectively. The two regions (Region 1 and Region 2) having less sequence similarity with that of the transgene 16cGFP mRNA (see Fig. 5), as well as nucleotide coordinates, are indicated.
Potential DCL2-Processed/Dependent siRNA Signals for Intercellular VIGS

In *Nb*, the DCL2-processed siRNAs (Figs. 4C and 5C) and/or DCL2-dependent siRNAs (produced by DCL2-activated pathways; Figs. 4E and 5E) are likely to be involved in the intercellular spread of epidermal cell-originating VIGS. Consistent with this idea, an elevated level of 22-nucleotide siRNAs was only found in TCV/GFPΔCP-inoculated *GfpDCL4Ai* and *GfpDCL4Bi* plants.

**Figure 5.** Distribution of 20- to 25-nucleotide GFP siRNAs across the 792-nucleotide transgene 16cGFP mRNA. A, *Gfp*. B, *GfpDCL1i*. C, *GfpDCL2Ai*. D, *GfpDCL3Bi*. E, *GfpDCL4Ai*. The sRNA libraries were generated from sRNA samples extracted from TCV/GFPΔCP-inoculated leaves. Blue and red bars represent siRNAs aligned to the sense (positive) and antisense (negative) strands of 16cGFP mRNA, respectively. The two regions (Region 1 and Region 2) having less sequence similarity with that of the transgene TcvGFP mRNA (see Fig. 4) as well as nucleotide coordinates are indicated.
that exhibited increased intercellular VIGS (Fig. 1; Supplemental Fig. S9). To examine this correlation further, we analyzed the size profiles of sense and antisense siRNA_{16cGFP} (Fig. 6). The 21-, 22-, and 24-nucleotide siRNA_{16cGFP} displayed similar size-profiles between Gfp and GfpDCL1i (Fig. 6, A and B, right panel). There was an obvious reduction in 24-nucleotide siRNA_{16cGFP} in GfpDCL3Bi (Fig. 6D, right panel). However, among Gfp, GfpDCL1i, and GfpDCL3Bi, the 21-nucleotide siRNA_{16cGFP} was always dominant whereas the levels of 22-nucleotide siRNAs remained similar (Fig. 6, A, B, and D, right panel; Supplemental Table S4). These findings further indicate that DCL1, DCL3, and DCL3-processed 24-nucleotide siRNAs may not significantly contribute to cell-to-cell spread of VIGS, consistent with the results of the local silencing assays (Fig. 1, H–U).

RNAi of DCL2 or DCL4 imposed contrasting effects on the accumulation of 21-, 22-, and 24-nucleotide siRNA_{16cGFP}. Compared to Gfp, GfpDCL1i, and GfpDCL3Bi (Fig. 6, A, B, and D, right panel), the absolute reads of 21-, 22-, and 24-nucleotide siRNA_{16cGFP} were reduced in GfpDCL2Ai (Fig. 6C, right panel), and were markedly increased in GfpDCL4Ai (Fig. 6E, right panel).

**Figure 6.** Size profiles of transgene GFP siRNA_{16cGFP}. A, Gfp. B, GfpDCL1i. C, GfpDCL2Ai. D, GfpDCL3Bi. E, GfpDCL4Ai. The sRNA libraries were generated from sRNA samples extracted from leaves with mock (left) or TCV/GFPΔCP (right) inoculation. Blue and red bars represent siRNAs aligned to the sense and antisense strands of the transgene 16cGFP mRNA, respectively.
Nonetheless, the percentage of the 22-nucleotide siRNA\textsubscript{16GFP} decreased in GfpDCL2Ai, whereas the relative abundance of 21-nucleotide siRNA\textsubscript{16GFP} was reduced in GfpDCL4Ai (Supplemental Table S4). These are in accordance with the respective roles of DCL4 and DCL2 in 21- and 22-nucleotide siRNA biosynthesis. The levels of DCL2-processed 22-nucleotide siRNA\textsubscript{16GFP} and DCL2-dependent siRNA\textsubscript{16GFP} were particularly low in GfpDCL2Ai (Fig. 6C, right panel), but copious in GfpDCL4Ai (Fig. 5E, right panel; Supplemental Table S4), consistent with the observed attenuation or enhancement of intercellular VIGS in the RNAi plants, respectively (Fig. 1). We also analyzed the size profiles of sense and antisense vsiRNA (Fig. 7). Distributions of 18-to 31-nucleotide vsiRNAs were not obviously altered among Gfp, GfpDCL1i, GfpDCL2Ai, and GfpDCL3Bi (Fig. 7, A–D, right panel). In these RNAi lines, the majority of vsiRNAs were 21 nucleotides in length (Fig. 7; Supplemental Table S4). However, in GfpDCL4Ai, vsiRNAs shifted their sizes largely to 22 nucleotides, although there were also marked increases in 21- and 24-nucleotide vsiRNAs (Fig. 7E, right panel). Notably, there was an approximate 10% reduction of 22-nucleotide vsiRNAs in GfpDCL2Ai compared to the

Figure 7. Size profiles of TCV/GFPΔCP viral siRNAs. A, Gfp. B, GfpDCL1i. C, GfpDCL2Ai. D, GfpDCL3Bi. E, GfpDCL4Ai. The sRNA libraries were generated from sRNA samples extracted from leaves with mock (left) or TCV/GFPΔCP (right) inoculation. Blue and red bars represent siRNAs aligned to the sense and antisense strands of TCV/GFPΔCP RNA, respectively.
as follows: (1) RNAi of the four

previous report that compromising of silencing ma-

viral functioning roles are debated (Bouché et al., 2006;

DCL2

2008).

are involved in cell-autonomous VIGS but their anti-

d CL2-processed/dependent mobile sig-

ns for intercellular VIGS. These conclusions are further

supported by similar results that were generated from six

extra sRNA libraries for the Gfp control and two different

RNAi lines GfpDCL2Bi and GfpDCL4Bi (Supplemental

Fig. S10, A–E).

DISCUSSION

In plants, DCLs play diverse roles in sense- and

hairpin-RNA-mediated PTGS and TGS (Parent et al.,

2015; Mlotshwa et al., 2008; Xie et al., 2004; 2005). DCL4,

DCL2, and their cognate 21- and 22-nucleotide vsiRNAs

are involved in cell-autonomous VIGS but their anti-

viral functioning roles are debated (Bouché et al., 2006;

Fusaro et al., 2006; Garcia-Ruiz et al., 2010; Qu et al.,

2008).

In this study, we reveal several interesting findings,

as follows: (1) RNAi of the four DCL genes does not

affect cell-to-cell movement deficiency of the immobile

virus TCV/GFPΔCP (Fig. 1). This is consistent with our

previous report that compromising of silencing machin-

ery alone was not sufficient to promote virus

movement (Shi et al., 2009). These findings ensure that

any intercellular VIGS that we observe in our assays do

not result from cell-to-cell movement of the recombi-

nant viral RNA, and also argue against the idea that an

altered ability to establish intra- and intercellular VIGS

in these DCL RNAi lines may enable TCV-GFPΔCP to

move locally or systemically more than in wild-type Nb

plants.

(2) DCL4 inhibited non-cell-autonomous intercellular

VIGS, whereas it acted as a major trigger for intracel-

lular VIGS (Fig. 3), consistent with its critical role in cell-

autonomous silencing and vsiRNA biogenesis (Wang

et al., 2011; Xie et al., 2005). Our findings are also in

agreement with previous reports that dcl4 mutations

enhance transitivity of cell-autonomous PTGS and can

rescue phloem-originating PTGS in Arabidopsis

(Mlotshwa et al., 2008; Parent et al., 2015). The fact that

DCL4 attenuates intercellular VIGS implies that DCL4-

processed 21-nucleotide vsiRNAs are unlikely to be

involved in cell-to-cell spread of VIGS in Nb.

(3) DCL2, probably along with DCL2-processed/

dependent siRNAs and their precursor RNAs, is in-

volved in intercellular VIGS. DCL2 was also able to

target and degrade viral RNAs in plant cells but this

activity was largely redundant when functional DCL4

was present (Fig. 3). These findings suggest that DCL2

could influence intracellular VIGS in Nb, although

DCL2 is thought to be dispensable for antiviral silencing

in Arabidopsis (Wang et al., 2011). Neither DCL1 nor

DCL3 affected vsiRNA production or intra- and inter-

cellular VIGS. Intriguingly, DCL2 played a key role in

spreading VIGS from individual epidermal cells to

adjacent epidermal and mesophyll cells—a formerly

unidentified function in silencing-based antiviral

defense.

(4) Silencing machinery degraded TcvGFP mRNA and

the resultant siRNA16cGFP targeted identical re-

gions in the transgene 16cGFP mRNA and generated

siRNA16cGFP for intracellular VIGS in TCV/GFPΔCP-

infected epidermal cells (Figs. 4 to 7). Such siRNA16cGFP

and siRNA16cGFP in sense and antisense polarities then

led to biogenesis of siRNAs associated with differ-

ent parts across the 16cGFP and TcvGFP RNA se-

quences for intra- and intercellular VIGS. Our results

thus imply that initial signals for intercellular VIGS

might consist of sense and antisense siRNA16cGFP and

siRNA16cGFP. Production of such signals in incipient

epidermal cells (i.e. the TCV/GFPΔCP-infected cells) and

subsequent induction of 16cGFP silencing in neighboring

recipient cells (i.e. TCV/GFPΔCP noninfected cells) were

influenced positively by DCL2, but negatively by DCL4
(Figs. 1 and 2). However, in contrast to complete loss-of-function genetic

mutants, RNAi lines are partial loss of function. It is also

possible that TCV/GFPΔCP infection could alter the

expression of the DCL genes targeted by RNAi. Se-

quenced small RNAs were from all of the cells in the

inoculated leaves, including the inducing and re-

ipient cells. Considering these factors, it remains

possible that long dsRNA precursors of DCL2 (or

DCL4) could move between cells or long distance for

induction of non-cell-autonomous VIGS.

Collectively, our results suggest that DCL4 and DCL2

play major but distinct roles in intra-/intercellular

VIGS. Involvement of DCL2 and DCL2-processed/

dependent siRNAs as well as their precursor RNAs in

intercellular VIGS is consistent with the fact that DCL2

and DCL2-processed 22-nucleotide siRNA can effec-

tively trigger biogenesis of secondary siRNAs in plants

(Chen et al., 2010), and restore intercellular PTGS in-

duced by sense- and hairpin-transgene RNAs in the

Arabidopsis dcl4 mutant (Mlotshwa et al., 2008; Parent

et al., 2015). It should be pointed out that silencing

spread in our system is more complex than other sys-

tems because it is dependent on the expression of both

p8 and p9 proteins of TCV (Zhou et al., 2008). Therefore,

we are cautious to expand our findings to other exam-

ples of cell-to-cell spread of RNA silencing, such as the

controversial Arabidopsis model in which, DCL4 and

DCL4-processed 21-nucleotide siRNAs are thought to

be directly involved in short-range cell-to-cell spread of

phloem-originating PTGS.

Nevertheless, our findings support a hypothesis that

DCL4 is essential for cell-autonomous intracellular

VIGS, but negatively regulates intercellular VIGS. This

is likely to be achieved via DCL4-mediated epistatic

interference over DCL2 because the latter is essential to

promote cell-to-cell spread of VIGS. Indeed DCL4 can

suppress the expression of DCL2 in Nb (Fig. 2). DCL2 is

also required to generate and perceive mobile signals

for systemic PTGS whereas DCL4 inhibits systemic

PTGS (unpublished data). To put these findings in the
context of RNA silencing-based defense, we propose two separate components of an integrated viral defense strategy in which DCL2 and DCL4 play different roles (Fig. 8). DCL4, the primary defender in the cell-autonomous intracellular VIGS, attacks viruses within the initially infected cells. Simultaneously it also inhibits non-cell-autonomous silencing. Thus, if this intracellular VIGS frontline in incipient cells was broken, for example through inhibiting DCL4 activity by VSR such as P1/HC-Pro and P38 (Csorba et al., 2015; Mlotshwa et al., 2008), intercellular VIGS would then be activated efficiently spreading to nearby recipient cells to form a second frontline against the virus. Non-cell-autonomous intercellular VIGS relies upon functional DCL2 and DCL2-processed/dependent siRNAs and their precursor RNAs. In this scenario, DCL2 is required to trigger the intercellular VIGS frontline and defend recipient cells from further virus invasion. DCL2 may also contribute to cell-autonomous VIGS, but DCL2 can only fulfill this activity when DCL4 is absent or dysfunctional. This explains why an increased intercellular VIGS was observed in DCL4 RNAi plants, but a decreased non-cell-autonomous VIGS was observed in DCL2 RNAi plants. Such a local dual-defense strategy would be more difficult for the virus to break down and may provide plants with an evolutionary advantage in their defense against viral pathogens (Supplemental Text S3).

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Wild-type Nicotiana benthamiana and transgenic lines (Supplemental Table S1) were grown and maintained in insect-free growth rooms at 25°C with supplementary lighting to give a 16-h photoperiod.

Figure 8. Cell- and non-cell autonomous VIGS in N. benthamiana. In incipient leaf epidermal cells (i.e. individual cells initially infected by TCV/GFPΔCP), DCL4 plays a critical role in biogenesis of vsiRNAs, siRNA_TcvGFP and transgene siRNA_16cGFP. These siRNAs are associated with cell-autonomous intracellular VIGS to inhibit local virus infection. DCL4-processed siRNAs are unlikely involved in spread of VIGS from leaf epidermal cell to adjacent cells because DCL4 inhibits intercellular VIGS. In the incipient cell, DCL2 can also target and dice viral RNAs, TcvGFP and 16cGFP mRNA into siRNAs, but this activity is largely blocked by DCL4 (T sign). In contrast, the key functionality of DCL2 is to trigger efficient intercellular VIGS. This is likely achieved through its activities to produce DCL2-processed/dependent siRNAs (and/or their precursor long RNAs, highlighted red) in incipient cells and to perceive these mobile signals for non-cell-autonomous intercellular VIGS in recipient epidermal and mesophyll cells. Neither DCL1 nor DCL3 affects vsiRNA production or intra- and intercellular VIGS. Thus DCL4 and DCL2 play major but distinct roles in cell- and non-cell-autonomous VIGS that form a dual antiviral frontline in incipient and recipient cells. DCL4, the primary defender for the cell-autonomous intracellular VIGS, can attack viruses within the initially infected cells. However, if viruses break through this defense frontline, non-cell-autonomous intracellular VIGS can efficiently spread to nearby recipient cells. This is due to loss of the negative control of intercellular VIGS mediated by DCL4. Intercellular VIGS is dependent upon functional DCL2 and DCL2-processed/dependent siRNAs (and/or their precursor long RNAs), but it is negatively controlled by DCL4. RNAi of DCL4 results in fully functional DCL2 that enhances cell-to-cell spread of VIGS. The intercellular VIGS can then defend recipient cells from further virus infection. Such a dual-defense strategy can compensate each other to give host cells evolutionary advantage to battle against virus infection. This model is relevant to virus-VIGS interaction at the intra-/intercellular level, rather than to systemic virus infection. The potential spread of DCL2-processed/dependent siRNAs (and their precursor long RNAs, highlighted in red) to move from the incipient to recipient cell through plasmodesmata is indicated with dashed arrows and cylinder signs. Inter-VIGS, intercellular VIGS; intra-VIGS, intracellular VIGS.
Plasmid Constructs, Virus Inoculation, and Microscopy

Construction of TCV/GFPΔCP was described by Ryabov et al. (2004). The full-length GFP sequence was PCR-amplified using TCV/GFPΔCP as a DNA template and cloned into pMD18-T (Takara) to produce a pT7.GFP construct from which GFP RNA transcripts were produced by in vitro transcription using T7 RNA polymerase. Primers used for making this construct are listed in Supplemental Table S1. TCV/GFPΔCP RNA was generated by in vitro transcription and used to mechanically inoculate N. D. D. RNAi, 16c.GFP, Gfp, and GfpDCL RNAi plants as described by Ryabov et al. (2004). Inoculated leaves were collected and visualized under an Axiopt microscope (Carl Zeiss) as described by Ryabov et al. (2004).

Intra- and Intercellular VIGS Assays

We used a cell-specific, silencing suppression-free and movement-deficient Turnip Crinkle Virus (TCV/GFPΔCP)–based system to induce intracellular VIGS in a single epidermal cell, from which silencing spreads to form visible silencing foci covering 100 to 300 epidermal cells, equivalent to a circular area with a radius of 6 to 10 epidermal cells, on the leaf epidermis of transgenic 16c.GFP plants (Qin et al., 2012; Ryabov et al., 2004; Zhou et al., 2008). Of important note, the precise location of a single epidermal cell that was initially infected with the movement-defective TCV/GFPΔCP could not be located before development of a visible silencing focus from the infected cell. Due to the compact TCV genome organization and viral gene expression strategy (Carrington et al., 1989; Cohen et al., 2000), it would be almost impossible to clone a second reporter gene, in addition to GFP, into TCV/GFPΔCP as an extra marker for measuring the initial infection of individual epidermal cell. Nevertheless, visible GFP silencing foci are a good indicator for induction and spread of TCV/GFPΔCP–induced intracellular VIGS. Upon mechanistic inoculation, their appearance is a gradual process starting from the individual cell on the upper epidermis, which is initially infected by TCV/GFPΔCP. Intracellular GFP silencing is induced by TCV/GFPΔCP in the single epidermal cell, and subsequently moves horizontally and vertically to neighboring upper epidermal, mesophyll, and lower epidermal cells in a three-dimensional manner, i.e. occurrence of intercellular VIGS (Qin et al., 2012; Zhou et al., 2008).

To perform intra- and intercellular VIGS assays, a single young leaf (second from top) of each of four to six seedlings (six-leaf stage) of 16c.GFP, Gfp, GfpDCL1i, GfpDCL2ai, GfpDCL2bi, GfpDCL3ai, GfpDCL3bi, or GfpDCL4bi lines were mechanically inoculated with an equal amount of RNA transcripts produced by in vitro transcription from 2.5 μg P7c-linearized TCV/GFPΔCP plasmid DNA, as described by Ryabov et al. (2004). Infection and spread of GFP silencing was routinely examined under a long-wavelength UV light and recorded photographically using a D7000 Digital Camera (Nikon). Regions of leaf epidermis in which silencing of GFP mRNA occurred showed red chlorophyll fluorescence, whereas tissues expressing GFP show green fluorescence under long-wavelength UV light. Numbers and sizes of GFP silencing foci (dark patches) were counted, measured, and photographed under an Axiopt microscope (Carl Zeiss) using settings to visualize GFP green fluorescence, as described by Qin et al. (2012). Number of silencing foci on an individual leaf was normalized against the average number of silencing foci per leaf of the control plants (i.e., 16c.GFP as control for GfpDCL1i, and Gfp as control for GfpDCL2ai, GfpDCL2bi, GfpDCL3ai, GfpDCL3bi, GfpDCL4ai, GfpDCL4bi, and GfpDCL4di) to minimize disparities that could be caused by experimental variations such as leaf size among different plants and freshly generated inoculum RNA transcripts used in different experiments. Silencing cell-to-cell spread index (SCI) was calculated as a percentage between the numbers of silencing foci counted on lower and upper (inoculated side) epidermis. Intra- and intercellular VIGS assays were performed for each of the transgenic lines in at least two separate experiments.

RNA Extraction and Northern Hybridization

For quantitative real-time PCR (qRT-PCR), total RNAs were extracted from leaf tissues using the RNAprep Pure Plant Kit (Tiangen) as recommended by the manufacturer. For northern blot, total RNAs were extracted from leaf tissues with Trizol reagent (Invitrogen) as recommended by the manufacturer. To analyze siRNAs, low-molecular-mass small RNAs were enriched from total RNA as described by Hamilton and Baulcombe (1999). The enriched small RNAs (2.5 μg) were fractionated on an 18% denaturing polyacrylamide-7 M urea gel in 1× Tris-borate-EDTA buffer. Small RNAs were transferred to Hybond-N membranes (Amersham Biosciences) by upward capillary transfer in 20× SSC buffer, then cross-linked to the membranes with a UVPC CX 2000 UV Crosslinker for four times (upside, underside, upside, underside) at 120 mJ/cm², 1 min each time. The membranes were hybridized with digoxigenin (Dig)-labeled GFP RNA probes prepared by in vitro transcription using the pT7.GFP and Dig RNA Labeling Kit (Roche) as recommended by the manufacturer. The hybridization chemiluminescence signals were detected with a ChemiDoc XR+ imaging system (Bio-Rad).

qRT-PCR

TCV/GFPΔCP or mock-inoculated leaves of N. d. D. RNAi, 16c.GFP, Gfp, and GfpDCL RNAi plants were taken at 7 dpi postinoculation (dpi) in three repeated experiments for RNA extraction. The first-strand 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 DCLs mRNA or TCV/GFPΔCP RNA levels were performed using specific primers (Supplemental Table S5) and the SYBR Green Mix. The amplification program for SYBR Green I was performed at 95°C for 10 s, 58°C for 30 s, and 72°C for 20 s on the CFX96 machine (Bio-Rad), following the manufacturer’s instructions. Quadruplicate quantitative assays (four technical replicates) were performed on cDNA of each of three to four biological duplicates (leaf tissues from three to four different treated plants). The relative RNA quantification was calculated using the expression 2 ΔΔCt and normalized to the amount of GAPDH (Gen-Bank accession no. TC17509) as described by Qin et al. (2012).

Construction of sRNA Library and sRNA Sequencing

Fragments of 18- to 30-bases-long RNA were isolated from total RNA extracted from mock- or TCV/GFPΔCP-inoculated leaf tissues of three to four different plants collected at 7 dpi after being separated through 15% denaturing PAGE. Then sRNAs were excised from the gel and sequentially ligated to 3' and 5'-adapters. After each ligation step, sRNAs were purified after 15% denaturing PAGE. The final purified ligation products were reversely transcribed into cDNA using reverse transcriptase (Finnzymes Oy). The first-strand cDNA was PCR-amplified using Phusion DNA Polymerase (Finnzymes Oy). The purified DNA fragments were used for clustering and sequencing by Illumina HiSeq 2000 (Illumina) at the Beijing Genomics Institute. It should be noted that a pool of leaves from three to four different plants was used for construction of each small RNA library. This avoided potential variations between individual treated plants, in particular those for TCV/GFPΔCP–based intra- and intercellular VIGS assays due to some variations of TCV/GFPΔCP replication in different plants.

Bioinformatics Analysis of sRNA Sequences

Illumina HiSeq 2000 sequencing produced 11 to 12 million reads per small RNA library. The reads were cropped to remove adapter sequences and were aligned to the reference sequences using the software Bowtie2 (Langmead and Salzberg, 2012; Ryabov et al., 2014). The reference sequences included TCV/GFPΔCP, viral TcGFP, and the 16c.GFP transgene (Haseloff et al., 1997; Ruiz et al., 1998; Ryabov et al., 2004); DCL1, DCL2, DCL3, and DCL4 gene sequences (Nakasugi et al., 2013); and the set of 50 tobacco microRNAs identified in Nicotiana plants (Nakasugi et al., 2014; Pandey et al., 2008). SAMtools pileup was produced to produce the siRNA and miRNA coverage profiles. For correlation analyses for the small RNA libraries, we determined numbers of the miRNA hits corresponding to the previously identified set of 50 Nicotiana miRNAs (Nakasugi et al., 2014; Pandey et al., 2008). All analyzed small RNA libraries contained similar proportions of host-encoded miRNA reads (Supplemental Data Sets S1–S3), indicating equivalence and direct comparability of the sRNA data sets. Indeed outcomes of comparisons between normalized sRNAs generated from target sequences against the total sRNA reads for all the libraries (per 10 million sRNA reads) are consistent with that the reads of siRNAs were directly compared.

Statistical Analysis

Normalized number of RNA silencing foci per leaf, sizes of RNA silencing foci, SCI, and qRT-PCR data between control and various treatments, were analyzed by Student’s t tests using an online program (http://www.physics.csbjos.edu/stats-t-test.html). It is worthwhile noting that ~4% or more change in the silencing foci sizes is of statistical significance due to the large numbers of samples (80–560) tested between wild-type controls and RNAi lines (Fig. 1; Supplemental Table S2).
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Supplemental Data

The following supplemental materials are available.

Supplemental Text S1. Parameters for assessing intra- and intercellular VIGS.

Supplemental Text S2. DCLs play differential roles in vsiRNA biogenesis.

Supplemental Text S3. Local VIGS versus virus interaction at the intra-/ 

intercellular level.

Supplemental Figure S1. Total small RNA profiles.

Supplemental Figure S2. Distribution of vsiRNAs and siRNA_{TcvGFP} across 

the TCV/GFPΔCP RNA.

Supplemental Figure S3. Distribution of vsiRNAs across the TCVΔCP RNA.

Supplemental Figure S4. Distribution of vsiRNAs across the TCVΔCP RNA.

Supplemental Figure S5. Impact of DCLi on TCV/GFPΔCP RNA replication.

Supplemental Figure S6. Comparisons between transgene 16cGFP and 

viral TcsGFP sequences.

Supplemental Figure S7. Distribution of 20- to 25-nucleotide GFP siRNAs 

across the 714-nucleotide TcsGFP mRNA.

Supplemental Figure S8. Distribution of 20- to 25-nucleotide GFP siRNAs 

across the 792-nucleotide transgene 16cGFP mRNA.

Supplemental Figure S9. Northern detection of TCV/GFPΔCP siRNAs.

Supplemental Figure S10. DCL2 and DCL2-dependent siRNAs for non-

cell-autonomous intercellular VIGS.

Supplemental Table S1. DCL RNAi lines used in this study.

Supplemental Table S2. Impact of DCL RNAi on cell-to-cell spread of 

virus-induced RNA silencing.

Supplemental Table S3. Summary of total viral and/or GFP siRNAs in mock- 

or TCV/GFPΔCP-inoculated leaves.

Supplemental Table S4. Percentage of 16cGFP and TCV/GFPΔCP 21-, 22-, 

and 24-nucleotide siRNA.

Supplemental Table S5. Primers used in this study.

Supplemental Data Set S1. Summary of siRNA and miRNA reads.

Supplemental Data Set S2. Correlation analyses of miRNA profiles among 

10 sRNA libraries.

Supplemental Data Set S3. Comparisons of miRNA among 10 sRNA libraries.

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