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1 Short Title: DCLs and siRNAs in Intra- and Intercellular VIGS 
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### 59 **ABSTRACT**

60 RNA silencing is an innate antiviral mechanism conserved in organisms across kingdoms.

- 61 Such a cellular defence involves DICER or DICER-LIKEs (DCLs) that process plant
- 62 virus RNAs into viral small interfering (vsi)RNAs. Plants encode four *DCLs* which play
- 63 diverse roles in cell-autonomous intracellular virus-induced RNA silencing (known as
- 64 VIGS) against viral invasion. VIGS can spread between cells. However, the genetic basis
- 65 and involvement of vsiRNAs in non-cell autonomous intercellular VIGS remains poorly
- 66 understood. Using GFP as a reporter gene together with a suite of *DCL* RNAi transgenic
- 67 lines, here we show that despite the well-established activities of *DCLs* in intracellular
- 68 VIGS and vsiRNA biogenesis, *DCL4* acts to inhibit intercellular VIGS whilst *DCL2* is
- 69 required (likely along with DCL2-processed/dependent vsiRNAs and their precursor
- 70 RNAs) for efficient intercellular VIGS trafficking from epidermal to adjacent cells.
- 71 *DCL4* imposed an epistatic effect on *DCL2* to impede cell-to-cell spread of VIGS. Our
- 72 results reveal previously unknown functions for *DCL2* and *DCL4* which may form a dual
- 73 defensive frontline for intra- and intercellular silencing to double-protect cells from virus
- 74 infection in *Nicotiana benthamiana*.
- 75
- 76 **Keywords:** DICER-LIKEs, vsiRNAs, non-cell autonomous intercellular VIGS,
- 77 *Nicotiana benthamiana*

#### 78 **INTRODUCTION**

79 RNA silencing targets endogenous cellular nucleic acids and exogenous invasive 80 pathogenic RNAs or DNAs for homologous RNA-dependent degradation, translation 81 repression or RNA-directed DNA methylation (RdDM) in eukaryotic organisms 82 (Baulcombe, 2004; Sarkies and Miska, 2014). In plants, RNA silencing forms an innate 83 defence against virus infection (Aliyari and Ding, 2009; Csorba et al., 2015). Such an 84 antiviral mechanism involves DICER-LIKE (DCL) ribonuclease type III enzymes. Most 85 plants encode four DCLs of which DCL1 is responsible for production of microRNA 86 whilst DCL2, DCL3 and DCL4 are responsible for biogenesis of 22, 24 and 21nt small 87 interfering RNA (siRNA), respectively (Mukherjee et al., 2013). DCL2 and DCL4 88 possess partially redundant functions in the production of trans-acting siRNA, but DCL2 89 acts predominantly to manufacture various sized secondary siRNAs (Chen et al., 2010; 90 Henderson et al., 2006; Xie et al., 2005). Unlike animal viruses, plant viruses have not 91 yet been found to encode any microRNA or specific site that can be targeted by host 92 cellular microRNAs. However, artificial microRNAs can inhibit plant virus invasion (Qu 93 et al., 2007). In *Arabidopsis*, DCLs can process plant virus RNAs into vsiRNAs within 94 individual cells. For instance, *DCL4* and DCL4-processed 21nt vsiRNAs are involved in 95 virus-induced RNA silencing (also known as VIGS), a kind of post-transcriptional gene 96 silencing (PTGS; Bouche et al., 2006; Garcia-Ruiz et al., 2010; Qu et al., 2008). *DCL2* 97 and its cognate 22nt vsiRNAs may also affect VIGS in plant cells when *DCL4* is absent 98 or defective (Andika et al., 2015; Wang et al., 2011; Zhang et al., 2012). On the other 99 hand, *DCL3* and 24nt vsiRNAs are associated with RdDM and transcriptional gene 100 silencing (TGS) in the protection of plant cells from DNA virus infection (Aregger et al., 101 2012; Blevins et al., 2006). In *Arabidopsis*, *DCL4* and *DCL2* also play hierarchical and 102 redundant roles in intracellular antiviral silencing (Bouche et al., 2006; Garcia-Ruiz et al., 103 2010; Wang et al., 2011). Recently, a combined activity of *DCL2* and *DCL3* has been 104 reported to be critical in defending plants from viroid infection (Katsarou et al., 2016). 105 DCL1 can negatively regulate DCL4-initiated antiviral RNA silencing pathway (Qu et al., 106 2008). However, the roles of the different *DCLs* in promoting intercellular VIGS for 107 plant systemic acquired resistance to virus infection are unclear.

108 In response to virus infection, intracellular VIGS in the initial virus-infected cells 109 triggers intercellular silencing in adjacent cells, which spreads systemically to remote 110 tissues. This is known as non-cell autonomous VIGS. Non-cell autonomous VIGS 111 combats incoming viruses and protects recipient cells from further viral invasion 112 (Schwach et al., 2005). In *Arabidopsis*, spread of the phloem-originating PTGS from 113 companion cells to nearby cells requires *DCL4* and DCL4-processed 21nt siRNA signals 114 (Dunoyer et al., 2005). However, whether 21nt siRNAs represent the bona fide silencing 115 signals that are transportable among plant cells is highly controversial (Berg, 2016). On 116 the other hand, *DCL2* can stimulate transitive PTGS and biogenesis of secondary siRNAs 117 (Mlotshwa et al., 2008). *DCL2* can also restore silencing in the *Arabidopsis dcl4* mutant 118 that is deficient in cell-to-cell spread of transgene-mediated PTGS (Parent et al., 2015). 119 Moreover, intercellular and systemic PTGS involve many cellular factors including 120 RDR6 which has been shown to be required for efficient cell-to-cell movement of VIGS 121 (Melnyk et al., 2011; Qin et al., 2012; Searle et al., 2010; Smith et al., 2007). 122 Nonetheless, the genetic basis and requirement of vsiRNAs for cell-to-cell and systemic 123 spread of antiviral VIGS remain to be elucidated. 124 We previously developed a Turnip crinkle virus (TCV)-based local silencing 125 assay to investigate intra- and intercellular VIGS in *Nicotiana benthamiana* (Qin et al., 126 2012; Ryabov et al., 2004; Shi et al., 2009; Zhou et al., 2008). TCV belongs to 127 *Carmovirus* with a single positive-stranded RNA genome (Carrington et al., 1989). It 128 encodes five proteins, namely the RNA-dependent RNA polymerases P28 and its read-129 through P88, movement proteins P8 and P9 and coat protein (CP) P38 (Carrington et al., 130 1989; Hacker et al., 1992; Li et al., 1998). CP is a strong viral suppressor of RNA 131 silencing (VSR; Chattopadhyay et al., 2015; Merai et al., 2006; Perez-Canamas et al., 132 2015; Qu et al., 2003; Thomas et al., 2003; Zhang et al., 2012). It is also required for cell-133 to-cell movement of TCV in *N. benthamiana* (Cohen et al., 2000; Li et al., 2009). 134 TCV/GFP∆CP in which CP is replaced with the 714nt GFP sequence (dubbed *TcvGFP* 135 hereafter) is movement-deficient. This movement-deficient virus is still infectious but the 136 virus remains restricted to the infected cell (Ryabov et al., 2004). Cell-to-cell spread of 137 TCV/GFP∆CP can be complemented by heterologous silencing suppressors (Shi et al., 138 2009). However in the absence of the strong VSR CP, the movement-deficient

- 139 TCV/GFP∆CP can initiate intracellular VIGS that efficiently spreads to neighbouring
- 140 epidermal and mesophyll cells (Qin et al., 2012; Ryabov et al., 2004; Shi et al., 2009;
- 141 Zhou et al., 2008). Using this intra- and intercellular VIGS assay together with a suite of
- 142 transgenic *DCL* RNAi lines, we have examined how the different *DCLs* affect viral
- 143 siRNA biogenesis, intra- and intercellular VIGS in *N. benthamiana*. Our findings lead us
- 144 to propose a model where intra- and intercellular VIGS comprise two separate
- 145 components of an integrated viral defence strategy in which *DCL2* and *DCL4* play
- 146 different roles.
- 147

148

#### 149 **RESULTS**

#### 150 *DCL* **RNAi Does Not Affect Cell-to-Cell Mobility of TCV/GFP∆CP**

151 To dissect the genetic requirements and silencing signals involved in non-cell

152 autonomous intercellular VIGS (Fig. 1) in *N. benthamiana* (*Nb*), we utilized a suite of

- 153 *DCL* RNAi transgenic *Nb* lines including *DCL1i*; *DCL2Ai* and *DCL2Bi*; *DCL3Ai* and
- 154 *DCL3Bi*; *DCL4Ai* and *DCL4Bi*; and one double RNAi line *DCL24i* (Supplemental Table
- 155 S1). We also used *GFP* transgenic lines *16cGFP*; *GfpDCL1i*; and lines *Gfp*; *GfpDCL2Ai*
- 156 and *GfpDCL2Bi*; *GfpDCL3Ai* and *GfpDCL3Bi*; *GfpDCL4Ai* and *GfpDCL4Bi*, which were
- 157 derived from crosses between *16cGFP* and *Nb* or *DCL* RNAi lines, respectively, as well
- 158 as a triple cross line *GfpDCL24i* (Supplemental Table S1). We performed qRT-PCR
- 159 assays and revealed that *DCL2, DCL3* and *DCL4* transcript levels were down-regulated
- 160 by 60-80% in each of the two independent RNAi lines; however only about 40%
- 161 reduction was achieved for *DCL1* in *DCL1i* (Fig. 1A). We then analyzed the impact of
- 162 *DCL* RNAi on cell-to-cell mobility of TCV/GFPΔCP (Fig. 1B-G). The upper epidermises
- 163 of leaves in each *DCL* RNAi plant at the six-leaf stage were inoculated with
- 164 TCV/GFP∆CP. As observed under the fluorescent microscope, strong GFP green
- 165 fluorescence appeared only in single epidermal cells in leaves of the wild-type *Nb* control
- 166 (Fig. 1C) and all *DCL* RNAi plants (Fig. 1D-G). These results demonstrate that presence
- 167 of TCV/GFP∆CP was limited to individual virus-infected epidermal cell, and that *DCL*

168 RNAi did not affect the movement-deficiency of TCV/GFP∆CP.

169

### 170 *DCL4* **RNAi Enhances, whilst** *DCL2* **RNAi Reduces, Cell-to-Cell Spread of VIGS**

- 171 To test whether intra- and intercellular VIGS is affected by the down-regulation of
- 172 individual *DCL* genes, we used GFP as a reporter and mechanically inoculated the
- 173 movement-deficient TCV/GFP∆CP onto young leaves of *16cGFP* (Fig. 1H), *GfpDCL1i*
- 174 (Fig. 1I), *Gfp* (Fig. 1J), *GfpDCL2Ai* and *GfpDCL2Bi* (Fig. 1K and L), *GfpDCL3Ai* and
- 175 *GfpDCL3Bi* (Fig. 1M and N) and *GfpDCL4Ai* and *GfpDCL4Bi* (Fig. 1O and P) plants.
- 176 We then counted the number of *GFP* silencing foci on both upper and lower epidermises
- 177 of the inoculated leaves, and measured sizes in diameter of 80-560 randomly selected
- 178 silencing foci on the upper epidermises (Fig. 1H-U; Supplemental Table S2). We used
- 179 the number and size of silencing foci as well as the 'silencing cell-to-cell-spread index



Figure 1. Different Roles of DCLs in the Cell-to-cell Spread of VIGS. (A) Downregulation of DCL expression by RNAi. Young leaves were collected from DCLi plants at 7 days post-inoculation (dpi), and the level of  $DCL$  RNAs was analysed by qRT-PCR. (B) Schematic of the intracellular RNA silencing trigger TCV/GFPACP. The T7 promoter, viral RNA-dependent RNA polymerases (P28, P88), movement proteins (P8 and P9) and GFP are indicated. (C-G) Restricted localization of TCV/GFPACP in single epidermal cell of Nb (C), DCLIi (D) DCL2Ai (E), DCL3Bi (F) and DCL4Ai (G) plants. (H-Q) Intercellular GFP silencing foci (dark patches indicated by red arrows). Photographs of silencing foci on leaves of 16cGFP (H); GfpDCL1i (I); Gfp (J); GfpDCL2Ai (K) and GfpDCL2Bi (L); GFPDCL3Ai (M) and GfpDCL3Bi (N); GfpDCL4Ai (O) and GfpDCL4Bi (P); and a triple-cross line  $GfpDCL24i$  (O), were taken under a fluorescent microscope at 7 dpi. Bar=500 $\mu$ m. (R) Normalized number of GFP silencing foci per upper epidermis. Silencing foci were counted at 7 dpi from 3-21 different plant leaves inoculated with TCV/GFPACP. (S) and (T) Average size (diameters, S) and percentage change (T) of silencing foci. 80-560 silencing foci on different upper epidermises were randomly selected and measured. (U) SCI calculated as percentage of number of silencing foci on lower epidermis out of the number of silencing foci on upper epidermis. Student's *t*-tests were performed for qRT-PCR and silencing data (mean  $\pm$  standard deviation) and P-values are indicated (asterisks).

- 180 (dubbed SCI hereafter)' to assess the influence of *DCL* RNAi on intra- and intercellular
- 181 VIGS (Supplemental Text S1). Compared to *16cGFP* and *Gfp* controls (Fig. 1H and J),
- 182 *DCL2* RNAi caused 17-22% decrease in the average sizes of silencing foci (Fig. 1K, L, S
- 183 and T; Supplemental Table S2). SCI was reduced from approximately 58% in *Gfp* plants

184 to 29-42% in *GfpDCL2Ai* and *GfpDCL2Bi* plants (Fig. 1U; Supplemental Table S2). 185 *DCL2* RNAi also caused a reduction in the number of silencing foci per leaf (Fig. 1R; 186 Supplemental Table S2). RNAi knock-down of *DCL1* or *DCL3* did not affect the number 187 of silencing foci and only reduced cell-to-cell movement of VIGS to a small extent, as 188 evidenced by 3-5% decreases in silencing foci sizes and/or some reductions in SCI (Fig. 189 1H-J, M, N and R-U; Supplemental Table S2). This suggests that *DCL3* and/or *DCL1* 190 may not contribute significantly to intercellular VIGS. A possible role cannot be ruled out 191 completely, however, due to discrepancy between the two *GfpDCL3i* lines, and for we 192 only have data from a single *GfpDCL1i* line. As with *DCL2* RNAi, *DCL4* RNAi caused a 193 reduction in the number of silencing foci in *GfpDCL4Ai* and *GfpDCL4Bi* plants (Fig. 1R; 194 Supplemental Table S2). This is consistent with the predominant role that *DCL4* plays in 195 intracellular VIGS. To our surprise, the average sizes of silencing foci increased by more 196 than 20% (Fig. 1O, P, S and T; Supplemental Table S2). The SCI also raised from around 197 58% in the *Gfp* controls to 70-75% in the two *GfpDCL4* RNAi lines (Fig. 1U; 198 Supplemental Table S2). These results demonstrate that *DCL4* RNAi reduced 199 intracellular silencing, but enhanced intercellular spread of VIGS. Taken together, our 200 findings show that *DCL4* RNAi enhances but *DCL2* RNAi reduces cell-to-cell spread of 201 VIGS in *Nb.*

# 202

### 203 *DCL4* **Interferes With** *DCL2* **to Control Intercellular VIGS**

- 204 To investigate whether *DCL4* and *DCL2* would affect each other to influence cell-to-cell
- 205 spread of VIGS in *Nb*, we inoculated the triple-cross *GfpDCL24i* plant with
- 206 TCV/GFP∆CP. We found a marked reduction in the number of *GFP* silencing foci (Fig.
- 207 1R; Supplemental Table S2), consistent with the reduction in the number of silencing foci
- 208 observed in *GfpDCL2* RNAi and *GfpDCL4* RNAi lines. However, in the triple-cross
- 209 plants, the average sizes of silencing foci decreased by more than 40%, and SCI also fell
- 210 from 58% to 44% when compared to the *Gfp* control (Fig. 1Q, S-T; Supplemental Table
- 211 S2). These results demonstrate that simultaneous RNAi of both *DCL2* and *DCL4* reduced
- 212 both intra- and intercellular VIGS, similar to what is seen in *GfpDCL2* RNAi lines but to
- 213 a greater extent. The inhibition of intercellular spread of VIGS in the triple-cross
- 214 *GfpDCL24i* line is opposite to the increase in intercellular VIGS seen in the *GfpDCL4*



Figure 2. Regulation of DCL2 Expression by DCL3 and DCL4. (A-C) Effects of RNAi of DCL2 (A), DCL3 (B) and DCL4 (C) on DCL gene expression. Young leaf tissues were collected at 6-8 leaf stage from four different plants of each transgenic line as indicated. RNA transcripts were analysed by qRT-PCR. Four technical replicates for qRT-PCR assays were performed on each cDNA of four biological duplicates  $(n = 4)$ ; leaf tissues from four different transgenic plants). Student's *t*-tests were performed for data (mean ± standard deviation) and P-values are indicated (asterisks). DCL2i does not affect expression of DCL1, DCL3 or DCL4 (A). However DCL3i (B) or DCL4i (C) resulted in increased mRNA levels of DCL2.

- 215 RNAi lines. This implies that *DCL4* imposed an epistatic effect on *DCL2* to affect
- 216 intercellular VIGS. This conclusion is supported by data from qRT-PCR assays (Fig. 2).
- 217 *DCL2* RNAi had no obvious impact on mRNA levels of *DCL1*, *DCL3* and *DCL4* (Fig.
- 218 2A). However, *DCL4* RNAi led to a 20–40% increase in *DCL2* expression but had no
- 219 substantial influence on the transcript levels of *DCL1* or *DCL3*; *DCL3* RNAi also
- 220 enhanced the level of *DCL2* transcripts, but did not affect expression of *DCL1* or *DCL4*
- 221 (Fig. 2B, C). Together with the specific RNAi effects on each *DCL* (Supplemental
- 222 Dataset S1), these data reveal that *DCL4* is involved in a negative regulation of *DCL2*
- 223 expression and as a consequence affecting the intercellular spread of VIGS in *Nb*.
- 224

#### 225 *DCLs* **Play Differential Roles in vsiRNA Biogenesis**

- 226 To further understand how *DCLs* contribute to intra- and intercellular VIGS, we
- 227 performed next generation sequencing of sRNA libraries for mock- or TCV/GFP∆CP-
- 228 inoculated *Gfp*, *GfpDCL1i*, *GfpDCL2Ai*, *GfpDCL3Bi* and *GfpDCL4Ai* (Supplemental
- 229 Text S2; Supplemental Dataset S1-3; Supplemental Fig. S1). We then mapped vsiRNAs
- 230 and *TcvGFP* siRNAs onto the sequence of TCV/GFP∆CP (Fig. 3; Supplemental Fig. S2)
- 231 and TCV∆CP (Supplemental Fig. S3-4). We found abundant vsiRNAs in TCV/GFP∆CP-
- 232 inoculated RNAi lines (Fig. 3A-E; Supplemental Fig. S3A-E), compared to their mock
- 233 controls (Supplemental Fig. S2A-E; Supplemental Fig. S4A-E; Supplemental Table S3).
- 234 This is consistent with induction of effective VIGS in these plants (Fig. 1H-U;
- 235 Supplemental Table S2). More vsiRNAs were recorded in *GfpDCL1i*, *GfpDCL2Ai* and
- 236 *GfpDCL3Bi* plants (Fig. 3B-D; Supplemental Fig. S3B-D) than in *Gfp* controls (Fig. 3A;
- 237 Supplemental Fig. S3A; Supplemental Table S3). However, the reads of vsiRNAs,
- 238 particularly in the sense polarity, decreased in *GfpDCL4Ai* plants (Fig. 3E; Supplemental
- 239 Fig. S3E; Supplemental Table S3) despite a marked increase in the overall number of
- 240 siRNAs (vsiRNAs and *TcvGFP*-siRNAs) mapped to TCV/GFP∆CP (Supplemental Table
- 241 S3). These results are consistent with the reduced level of recombinant viral RNAs in
- 242 TCV/GFP∆CP-inoculated *DCL* RNAi plants, compared to the non-RNAi controls
- 243 (Supplemental Fig. S5). On the other hand, the distribution of vsiRNAs across
- 244 TCV/GFP∆CP (Fig. 3A-E) or TCV∆CP (Supplemental Fig. S3A-E) was identical among
- 245 all virus-inoculated RNAi and control plants. Taken together, these data demonstrate that
- 246 *DCL4* is able to efficiently target viral RNAs for the production of vsiRNAs during cell-
- 247 autonomous VIGS. Our results also reveal that *DCL2* is required for cell-to-cell spread of
- 248 VIGS, and *DCL2* could target viral RNA and *TcvGFP* mRNA for degradation in *Nb*.
- 249



RNA genome organization of TCV/GFP∆CP (4221 nt)

Figure 3. Distribution of 20-25nt vsiRNAs and siRNA $_{\rm{ToGFP}}$  across the TCV/GFP $\Delta$ CP RNA. (A) Gfp. (B) GfpDCL1i. (C) GfpDCL2Ai. (D) GfpDCL3Bi. (E) GfpDCL4Ai. The sRNA libraries were generated from sRNA samples extracted from TCV/GFPACPinoculated leaves. Blue and red bars represent siRNAs aligned to the sense (positive) and antisense (negative) strands of TCV/GFPACP viral RNA and TcvGFP mRNA (highlighted), respectively. The TCV/GFPACP genome organisation is indicated.

# 250 **Antagonistic Influences of** *DCL4* **and** *DCL2* **on Accumulation of siRNAs Associated**

#### 251 **with Intercellular VIGS**

- 252 In contrast to the situation with vsiRNAs, *GfpDCL* RNAi lines differed in the generation
- 253 of  $TcvGFP$  or transgene  $16cGFP$  siRNAs (dubbed siRNA<sub>TcvGFP</sub> and siRNA<sub>16cGFP</sub>) that are



mRNA. (A) Gfp. (B) GfpDCL1i. (C) GfpDCL2Ai. (D) GfpDCL3Bi. (E) GfpDCL4Ai. The sRNA libraries were generated from sRNA samples extracted from TCV/GFPACPinoculated 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.

- 254 associated with intra- and intercellular VIGS. Note that the 714nt *TcvGFP* (Ryabov et al.,
- 255 2004) and 792nt *16cGFP* (Haseloff et al., 1997; Ruiz et al., 1998) mRNAs are not
- 256 identical. Sequences between nucleotides 237-306 and 345-540 in *TcvGFP* (designated
- 257 TcvGFP<sub>237-306</sub> and TcvGFP<sub>345-540</sub>) differ from the corresponding regions 300-369 and



Figure 4. Distribution of 20-25nt GFP siRNAs across the 714nt TcvGFP mRNA. (A) Gfp. (B) GfpDCLIi. (C) GfpDCL2Ai. (D) GfpDCL3Bi. (E) GfpDCL4Ai. The sRNA libraries were generated from sRNA samples extracted from TCV/GFPACP-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.

- 258 408-603 in *16cGFP* (designated 16cGFP<sub>300-369</sub> and 16cGFP<sub>408-603</sub>) (Supplemental Fig. S6).
- 259 Compared to the *Gfp* control and *GfpDCL1i* and *GfpDCL3Bi* plants, the levels of
- 260 siRNATcvGFP and siRNA16cGFP were reduced in *GfpDCL2Ai*, but significantly increased in
- 261 *GfpDCL4Ai* (Supplemental Table S3). We then mapped the siRNAs onto *TcvGFP* (Fig. 4;

262 Supplemental Fig. S7) and *16cGFP* mRNA (Fig. 5; Supplemental Fig. S8). The 263 distribution of sense and antisense *GFP* siRNAs to regions that are identical in *TcvGFP* 264 and *16cGFP* was essentially the same in *Gfp* and in each of the *DCL* RNAi lines, 265 however, the levels of  $siRNA_{TcvGFP}$  and  $siRNA_{16cGFP}$  were lower in  $GfpDCL2Ai$ , and 266 much higher in *GfpDCL4Ai*, compared to *Gfp*, *GfpDCL1i* and *GfpDCL3Bi* (Fig. 4A-E; 267 Fig. 5A-E; Supplemental Table S3). Moreover in *GfpDCL4Ai* the level of siRNA<sub>16cGFP</sub> 268 (2.5 million reads) was approximately double compared to the abundance of si $\text{RNA}_{\text{ToVGFP}}$ 269 (1.28 million reads) (Supplemental Table S3). Such substantial differences between 270 siRNATcvGFP and siRNA16cGFP levels suggest that the transgene *16cGFP* mRNA was 271 targeted and diced by intra- and intercellular VIGS to a greater extent than *TcvGFP* 272 transcripts. In contrast, different profiles were observed for  $siRNA_{TcvVGFP}$  and 273 siRNA<sub>16cGFP</sub> corresponding to the two less-similar regions (Region 1: TcvGFP<sub>237-306</sub> and 274 16cGFP<sub>300-369</sub>; Region 2: TcvGFP<sub>345-540</sub> and 16cGFP<sub>408-603</sub>) (Fig. 4A-E; Fig. 5A-E). 275 TcvGFP<sub>237-306</sub> and TcvGFP<sub>345-540</sub> siRNAs were of low abundance and generally of sense 276 polarity in the control and all RNAi lines (Fig. 4A-E). However, higher levels of  $277$  16cGFP<sub>300-369</sub> and 16cGFP<sub>408-603</sub> siRNAs were observed, a significant amount of which 278 was antisense. As with the other GFP siRNAs, the levels of  $16cGFP<sub>300-369</sub>$  and  $16cGFP<sub>408-369</sub>$ 279 603 siRNAs were much higher in *GfpDCL4Ai* and lower in *GfpDCL2Ai*, compared to *Gfp*, 280 *GfpDCL1i* and *GfpDCL3Bi* (Fig. 5A-E). 281 These results demonstrate that *DCL4* and *DCL2* antagonistically affected the 282 accumulation of siRNAs associated with intercellular VIGS. The reduction of 283 siRNATcvGFP and siRNA16cGFP in *GfpDCL2Ai* or the massive accumulation of these 284 siRNAs in *GfpDCL4Ai* is likely to be due to the respective loss or gain-of-function of 285 DCL2-dependent production of primary or secondary siRNAs in these RNAi lines. 286 287 **Potential DCL2-processed/dependent siRNA Signals for Intercellular VIGS**  288 In *Nb*, the DCL2-processed siRNAs (Fig. 4C; Fig. 5C) and/or DCL2-dependent siRNAs 289 (produced by *DCL2-*activated pathways; Fig. 4E; Fig. 5E) are likely to be involved in the 290 intercellular spread of epidermal cell-originating VIGS. Consistent with this idea, an

- 291 elevated level of 22nt siRNAs was only found in TCV/GFP∆CP-inoculated *GfpDCL4Ai*
- 292 and *GfpDCL4Bi* plants that exhibited increased intercellular VIGS (Fig. 1; Supplemental



Figure 6. Size Profiles of Transgene GFP siRNA<sub>16cGFP</sub>. (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/GFPACP (right) inoculation. Blue and red bars represent siRNAs aligned to the sense and antisense strands of the transgene *16cGFP* mRNA respectively.

- 293 Fig. S9). To examine this correlation further, we analyzed the size profiles of sense and
- 294 antisense siRNA<sub>16cGFP</sub> (Fig. 6). The 21, 22 and 24nt siRNA<sub>16cGFP</sub> displayed similar size-
- 295 profiles between *Gfp* and *GfpDCL1i* (Fig. 6A and B, right panel). There was an obvious
- 296 reduction in 24nt siRNA16cGFP in *GfpDCL3Bi* (Fig. 6D, right panel). However among *Gfp*,

297 *GfpDCL1i* and *GfpDCL3Bi*, the 21nt siRNA16cGFP was always dominant while the levels

- 298 of 22nt siRNAs remained similar (Fig. 6A, B and D, right panel; Supplemental Table S4).
- 299 These findings further indicate that *DCL1*, *DCL3*, and DCL3-processed 24nt siRNAs
- 300 may not significantly contribute to cell-to-cell spread of VIGS, consistent with the results 301 of the local silencing assays (Fig. 1H-U).
- 302 RNAi of *DCL2* or *DCL4* imposed contrasting effects on the accumulation of 21, 303 22 and 24nt siRNA16cGFP. Compared to *Gfp*, *GfpDCL1i* and *GfpDCL3Bi* (Fig. 6A, B and 304 D, right panel), the absolute reads of 21, 22 and 24nt siRNA16cGFP reduced in *GfpDCL2Ai*  305 (Fig. 6C, right panel), and markedly increased in *GfpDCL4Ai* (Fig. 6E, right panel). 306 Nonetheless, the percentage of the 22nt siRNA16cGFP decreased in *GfpDCL2Ai* whilst the 307 relative abundance of 21nt siRNA16cGFP reduced in *GfpDCL4Ai* (Supplemental Table S4). 308 These are in accordance with the respective roles of DCL4 and DCL2 in 21 and 22nt  $309$  siRNA biosynthesis. The levels of DCL2-processed 22nt siRNA<sub>16cGFP</sub> and DCL2-310 dependent siRNA16cGFP were particularly low in *GfpDCL2Ai* (Fig. 6C, right panel), but 311 copious in *GfpDCL4Ai* (Fig. 5E, right panel; Supplemental Table S4), consistent with the 312 observed attenuation or enhancement of intercellular VIGS in the RNAi plants 313 respectively (Fig. 1). We also analyzed the size profiles of sense and antisense vsiRNA 314 (Fig. 7). Distributions of 18–31-nt vsiRNAs were not obviously altered among *Gfp*, 315 *GfpDCL1i*, *GfpDCL2Ai* and *GfpDCL3Bi* (Fig. 7A-D, right panel). In these RNAi lines, 316 the majority of vsiRNAs were 21nt in length (Fig. 7; Supplemental Table S4). However 317 in *GfpDCL4Ai*, vsiRNAs shifted their sizes largely to 22nt although there were also 318 marked increases in 21 and 24nt vsiRNAs (Fig. 7E, right panel). Notably, there was an 319 approximate 10% reduction of 22-nt vsiRNAs in *GfpDCL2Ai* compared to the *Gfp* 320 control (Supplemental Table S4). Taken together, our data show that *DCL4* plays a major, 321 and *DCL2* a minor, role in producing 21 or 22nt vsiRNAs for intracellular VIGS, whilst 322 *DCL2* is required to generate and perceive DCL2-processed/dependent mobile signals for 323 intercellular VIGS. These conclusions are further supported by similar results that were 324 generated from six extra sRNA libraries for the *Gfp* control and two different RNAi lines 325 *GfpDCL2Bi* and *GfpDCL4Bi* (Supplemental Fig. S10A-E). 326
- 327



Figure 7. Size Profiles of TCV/GFP∆CP 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/GFPACP (right) inoculation. Blue and red bars represent siRNAs aligned to the sense and antisense strands of TCV/GFPACP RNA, respectively.

# 328 **DISCUSSION**

- 329 In plants, *DCLs* play diverse roles in sense- and hairpin RNA-mediated PTGS and TGS
- 330 (Parent et al., 2015; Mlotshwa et al., 2008; Xie et al., 2004; 2005). *DCL4*, *DCL2* and their
- 331 cognate 21 and 22nt vsiRNAs are involved in cell-autonomous VIGS but their antiviral

332 functioning roles are debated (Bouche et al., 2006; Fusaro et al, 2006; Garcia-Ruiz et al, 333 2010; Qu et al., 2008).

334 In this study, we reveal several interesting findings:

335 (1) RNAi of the four *DCL* genes does not affect cell-to-cell movement deficiency 336 of the immobile virus TCV/GFP∆CP (Fig.1). This is consistent with our previous report 337 that compromising of silencing machinery alone was not sufficient to promote virus 338 movement (Shi et al., 2009). These findings ensure that any intercellular VIGS that we 339 observe in our assays do not result from cell-to-cell movement of the recombinant viral 340 RNA, and also argue against the idea that an altered ability to establish intra- and 341 intercellular VIGS in these *DCL* DNAi lines may enable TCV-GFPΔCP to move locally 342 or systemically more than in wild-type *Nb* plants.

343 (2) *DCL4* inhibited non-cell autonomous intercellular VIGS whilst it acted as a 344 major trigger for intracellular VIGS (Fig. 3), consistent with its critical role in cell-345 autonomous silencing and vsiRNA biogenesis (Wang et al., 2011; Xie et al., 2005). Our 346 findings are also in agreement with previous reports that *dcl4* mutations enhance 347 transitivity of cell-autonomous PTGS and can rescue phloem-originating PTGS in 348 *Arabidopsis* (Mlotshwa et al., 2008; Parent et al., 2015). The fact that *DCL4* attenuates 349 intercellular VIGS implies that DCL4-processed 21nt vsiRNAs are unlikely to be 350 involved in cell-to-cell spread of VIGS in *Nb*.

351 (3) *DCL2*, probably along with DCL2-processed/dependent siRNAs and their 352 precursor RNAs, is involved in intercellular VIGS. *DCL2* was also able to target and 353 degrade viral RNAs in plant cells but this activity was largely redundant when functional 354 *DCL4* was present (Fig. 3). These findings suggest that *DCL2* could influence 355 intracellular VIGS in *Nb* although *DCL2* is thought to be dispensable for antiviral 356 silencing in *Arabidopsis* (Wang et al., 2011). Neither *DCL1* nor *DCL3* affected vsiRNA 357 production or intra- and intercellular VIGS. Intriguingly, *DCL2* played a key role in 358 spreading VIGS from individual epidermal cell to adjacent epidermal and mesophyll cells, 359 a formerly unidentified function in silencing-based antiviral defense. 360 (4) Silencing machinery degraded  $TcvGFP$  mRNA and the resultant siRNA<sub>TcvGFP</sub> 361 targeted identical regions in the transgene *16cGFP* mRNA and generated siRNA<sub>16cGFP</sub> for

362 intracellular VIGS in TCV/GFP∆CP-infected epidermal cells (Figs. 4-7). Such

 $363$  siRNA<sub>TcvGFP</sub> and siRNA<sub>16cGFP</sub> in sense and antisense polarities then led to biogenesis of 364 siRNAs associated with different parts across the *16cGFP* and *TcvGFP* RNA sequences 365 for intra- and intercellular VIGS. Our results thus imply that initial signals for 366 intercellular VIGS might consist of sense and antisense si $\rm RNA_{TevGFP}$  and si $\rm RNA_{16cGFP}$ . 367 Production of such signals in incipient epidermal cell (*i.e.* the TCV/GFP∆CP-infected 368 cell) and subsequent induction of *16cGFP* silencing in neighboring recipient cells (*i.e.*  369 TCV/GFP∆CP non-infected cells) were influenced positively by *DCL2*, but negatively by 370 *DCL4* (Fig. 1; Fig. 2). However, in contrast to complete loss-of-function genetic mutants, 371 RNAi lines are partial loss of function. It is also possible that TCV/GFP∆CP infection 372 could alter the expression of the *DCL* genes targeted by RNAi. Sequenced small RNAs 373 were from all of the cells in the inoculated leaves, including the inducing and recipient 374 cells. Considering these factors, it remains possible that long dsRNA precursors of DCL2 375 (or DCL4) could move between cells or long distance for induction of non-cell 376 autonomous VIGS.

377 Collectively, our results suggest that *DCL4* and *DCL2* play major but distinct 378 roles in intra-/intercellular VIGS. Involvement of *DCL2* and DCL2-processed/dependent 379 siRNAs as well as their precursor RNAs in intercellular VIGS is consistent with the fact 380 that DCL2 and DCL2-processed 22nt siRNA can effectively trigger biogenesis of 381 secondary siRNAs in plants (Chen et al., 2010), and restore intercellular PTGS induced 382 by sense- and hairpin-transgene RNAs in the *Arabidopsis dcl4* mutant (Mlotshwa et al., 383 2008; Parent et al., 2015). It should be pointed out that silencing spread in our system is 384 more complex than other systems since it is dependent on the expression of both p8 and 385 p9 proteins of TCV (Zhou et al., 2008). Therefore, we are cautious to expand our findings 386 to other examples of cell-to-cell spread of RNA silencing, such as the controversial 387 *Arabidopsis* model in which, *DCL4* and DCL4-processed 21-nt siRNAs are thought to be 388 directly involved in short-range cell-to-cell spread of phloem-originating PTGS. 389 Nevertheless, our findings support a hypothesis that *DCL4* is essential for cell-

390 autonomous intracellular VIGS, but negatively regulates intercellular VIGS. This is likely 391 to be achieved via *DCL4*-mediated epistatic interference over *DCL2* because the latter is 392 essential to promote cell-to-cell spread of VIGS. Indeed *DCL4* can suppress the 393 expression of *DCL2* in *Nb* (Fig. 2). *DCL2* is also required to generate and perceive



Figure 8. Cell- and Non-cell Autonomous VIGS in N. benthamiana. In incipient leaf epidermal cells (i.e. individual cells initially infected by TCV/GFPACP), DCL4 plays a critical role in biogenesis of vsiRNAs, siRNATCVGFP and transgene siRNA<sub>16cGFP</sub>. These siRNAs are associated with cell-autonomous intracellular VIGS (Intra-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 (Inter-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 inter-VIGS in recipient epidermal and mesophyll cells. Neither DCL1 nor DCL3 affects vsiRNA production, 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 defence 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 DCL2processed/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-tocell spread of VIGS. The intercellular VIGS can then defend recipient cells from further virus infection. Such a dual-defence 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 red) to move from the incipient to recipient cell through plasmodesmata is indicated with dashed arrows and cylinder signs.

- 394 mobile signals for systemic PTGS whilst *DCL4* inhibits systemic PTGS (Fan et al., 2017,
- 395 under revision). To put these findings in the context of RNA silencing-based defence, we
- 396 propose two separate components of an integrated viral defence strategy in which *DCL2*
- 397 and *DCL4* play different roles (Fig. 8). *DCL4*, the primary defender in the cell-

398 autonomous intracellular VIGS, attacks viruses within the initially infected cells. 399 Simultaneously it also inhibits non-cell autonomous silencing. Thus, if this intracellular 400 VIGS frontline in incipient cells was broken, for example through inhibiting *DCL4* 401 activity by VSR such as P1/HC-Pro and P38 (Csorba et al., 2015; Mlotshwa et al, 2008), 402 intercellular VIGS would then be activated efficiently spreading to nearby recipient cells 403 to form a second frontline against the virus. Non-cell autonomous intercellular VIGS 404 relies upon functional *DCL2* and DCL2-processed/dependent siRNAs and their precursor 405 RNAs. In this scenario, *DCL2* is required to trigger the intercellular VIGS frontline and 406 defend recipient cells from further virus invasion. *DCL2* may also contribute to cell-407 autonomous VIGS, but *DCL2* can only fulfil this activity when *DCL4* is absent or 408 dysfunctional. This explains why an increased intercellular VIGS was observed in *DCL4* 409 RNAi plants, but a deceased non-cell autonomous VIGS in *DCL2* RNAi plants. Such a 410 local dual-defence strategy would be more difficult for the virus to breakdown and may 411 provide plants with an evolutionary advantage in their defence against viral pathogens 412 (Supplemental Text S3).

413

# 414 **MATERIALS AND METHODS**

# 415 **Plant Materials and Growth Conditions**

416 Wild-type *Nicotiana benthamiana* (*Nb*) and transgenic lines (Supplemental Table S1)

417 were grown and maintained in insect-free growth-rooms at 25℃ with supplementary

- 418 lighting to give a 16-hour photoperiod.
- 419

# 420 **Plasmid Constructs, Virus Inoculation and Microscopy**

421 Construction of TCV/GFPΔCP was previously described (Ryabov et al., 2004). The full-

422 length GFP sequence was PCR amplified using TCV/GFPΔCP as DNA template and

- 423 cloned into pMD18-T (Takara) to produce pT7.GFP construct from which *GFP* RNA
- 424 transcripts were produced by *in vitro* transcription using T7 RNA polymerase. Primers
- 425 used for making this construct are listed in Supplemental Table S5. TCV/GFPΔCP RNA
- 426 was generated by *in vitro* transcription and used to mechanically inoculate *Nb*, *DCL*
- 427 RNAi, *16cGFP*, *Gfp*, *GfpDCL* RNAi plants as described (Ryabov et al., 2004).

428 Inoculated leaves were collected and visualized under a Zeiss Axiphot microscope as 429 described (Ryabov et al., 2004).

430

#### 431 **Intra- and Intercellular VIGS Assays**

432 We used a cell-specific, silencing suppression-free and movement-deficient Turnip 433 crinkle virus (TCV/GFP∆CP)-based system to induce intracellular VIGS in a single 434 epidermal cell, from which silencing spreads to form visible silencing foci covering 100- 435 300 epidermal cells, equivalent to a circular area with a radius of 6-10 epidermal cells, on 436 the leaf epidermis of transgenic *16cGFP* plants (Qin et al., 2012; Ryabov et al., 2004; 437 Zhou et al., 2008). Of important notes, the precise location of a single epidermal cell that 438 was initially infected with the movement-defective TCV/GFP∆CP could not be located 439 prior to development of a visible silencing focus from the infected cell. Due to the 440 compact TCV genome organization and viral gene expression strategy (Carrington et al., 441 1989; Cohen et al., 2000), it would be almost impossible to clone a second reporter gene, 442 in addition to *GFP*, into TCV/GFP∆CP as an extra marker for measuring the initial 443 infection of individual epidermal cell. Nevertheless, visible *GFP* silencing foci are a good 444 indicator for induction and spread of TCV/GFP∆CP-induced intracellular VIGS. Upon 445 mechanic inoculation, their appearance is a gradual process starting from the individual 446 cell on the upper epidermises which is initially infected by TCV/GFP∆CP. Intracellular 447 *GFP* silencing is induced by TCV/GFP∆CP in the single epidermal cell; and 448 subsequently moves horizontally and vertically to neighboring upper epidermal, 449 mesophyll and lower epidermal cells in a three-dimensional manner, i.e. occurrence of 450 intercellular VIGS (Qin et al., 2012; Zhou et al., 2008). 451 To perform intra- and intercellular VIGS assays, a single young leaf  $(2<sup>nd</sup>$  from top) 452 of each of four-to-six seedlings (six-leaf stage) of *16cGFP*, *Gfp GfpDCL1i*, *GfpDCL2Ai*, 453 *GfpDCL2Bi*, *GfpDCL3Ai*, *GfpDCL3Bi*, *GfpDCL4Ai* or *GfpDCL4Bi* lines were 454 mechanically inoculated with an equal amount of RNA transcripts produced by *in vitro* 455 transcription from 2.5-µg *Pac*I-linearized TCV/GFPΔCP plasmid DNA as described 456 (Ryabov et al., 2004). Induction and spread of *GFP* silencing was routinely examined 457 under long-wavelength UV light and recorded photographically using a Nikon Digital

458 Camera D7000. Regions of leaf lamina in which silencing of *GFP* mRNA occurred show

- 459 red chlorophyll fluorescence, while tissues expressing GFP show green fluorescence
- 460 under long-wavelength UV light. Numbers and sizes of *GFP* silencing foci (dark patches)
- 461 were counted, measured and photographed under a Zeiss Axiphot microscope using
- 462 settings to visualize GFP green fluorescence as described (Qin et al., 2012). Number of
- 463 silencing foci on an individual leaf was normalized against the average number of
- 464 silencing foci per leaf of the control plants (i.e. *16cGFP* as control for *GfpDCL1i*, and
- 465 *Gfp* as control for *GfpDCL2Ai*, *GfpDCL2Bi*, *GfpDCL3Ai*, *GfpbDCL3Bi*, *GfpDCL4Ai*,
- 466 *GfpDCL4Bi* and *GfpDCL24i*) to minimize disparities that could be caused by
- 467 experimental variations such as leaf sizes among different plants and freshly-generated
- 468 inoculum RNA transcripts used in different experiments. SCI was calculated as
- 469 percentage between the numbers of silencing foci counted on lower and upper (inoculated
- 470 side) epidermises. Intra- and intercellular VIGS assays were performed for each of the
- 471 transgenic lines in at least two separate experiments.
- 472

### 473 **RNA Extraction and Northern Hybridization**

474 For quantitative Real-Time PCR (qRT-PCR), total RNAs were extracted from leaf tissues

- 475 using the RNAprep Pure Plant Kit (Tiangen) as recommended by the manufacturer. For
- 476 Northern blot, total RNAs were extracted from leaf tissues with Trizol reagent
- 477 (Invitrogen, Carlsbad, CA) as recommended by the manufacturer. To analyze siRNAs,
- 478 low-molecular-mass small RNAs were enriched from total RNA as previously described
- 479 (Hamilton and Baulcombe, 1999). The enriched small RNAs (2.5μg) were fractionated
- 480 on an 18% denaturing polyacrylamide–7 M urea gel in 1 x Tris–borate–EDTA (TBE)
- 481 buffer. Small RNAs were transferred to Hybond-N+ membranes (Amersham
- 482 Biosciences) by upward capillary transfer in 20xSSC buffer, then cross-linked to the
- 483 membranes with an UVP CX 2000 UV Crosslinker for 4 times (upside, underside, upside,
- 484 underside) at 120 millijoules/cm<sup>2</sup>, 1 minute each time. The membranes were hybridized
- 485 with digoxigenin (Dig)-labelled *GFP* RNA probes prepared by in vitro transcription
- 486 using pT7.GFP and DIG RNA Labeling Kit (Roche) as recommended by the
- 487 manufacturer. The hybridization chemiluminescence signals were detected with a
- 488 ChemiDoc<sup>TM</sup> XRS+ imaging System (Bio Rad).
- 489

#### 490 **qRT-PCR**

- 491 TCV/GFP∆CP or mock-inoculated leaves of *Nb*, *DCL* RNAi, *16cGFP, Gfp*, and *GfpDCL*
- 492 RNAi plants were taken at 7 days post inoculation in three repeated experiments for RNA
- 493 extraction. The first-stranded cDNA was synthesized using total RNAs treated with
- 494 RNase-free DNase I as templates by the M-MLV Reverse Transcriptase (Promega). The
- 495 qRT–PCR analyses of *DCLs* mRNA or TCV/GFP∆CP RNA levels were performed using
- 496 specific primers (Supplemental Table S5) and the SYBR Green Mix, The amplification
- 497 program for SYBR Green I was performed at 95<sup>o</sup>C for 10 seconds, 58<sup>o</sup>C for 30 seconds
- 498 and 72<sup>o</sup>C for 20 seconds on the CFX96 machine (Bio-Rad), following the manufacturer's
- 499 instructions. Quadruplicate quantitative assays (four technical replicates) were performed
- 500 on cDNA of each of three-four biological duplicates (leaf tissues from three-four different
- 501 treated plants). The relative RNA quantification was calculated using the formula  $2^{-\Delta\Delta Ct}$
- 502 and normalized to the amount of GAPDH (Genbank accession number TC17509) as
- 503 described (Qin et al., 2012).
- 504

#### 505 **Construction of sRNA Library and sRNA Sequencing**

506 Fragments of 18-30 bases long RNA were isolated from total RNA extracted from mock-507 or TCV/GFP∆CP-inoculated leaf tissues of 3-4 different plants collected at seven days 508 post-inoculation (dpi) after being separated through 15% denaturing PAGE. Then sRNAs 509 were excised from the gel and sequentially ligated to 3'- and 5'-adapters. After each 510 ligation step, sRNAs were purified after 15% denaturing PAGE. The final purified 511 ligation products were reversely transcribed into cDNA using reverse transcriptase 512 (Finnzymes Oy). The first strand cDNA was PCR amplified using Phusion\* DNA 513 Polymerase (Finnzymes Oy). The purified DNA fragments were used for clustering and 514 sequencing by Illumina Hiseq 2000 (Illumina, San Diego, CA) at the Beijing Genomics 515 Institute, Shenzhen. It should be noted that a pool of leaves from 3-4 different plants was 516 used for construction of each sRNA library. This avoided potential variations between 517 individual treated plants, in particular these for TCV/GFP∆CP-based intra- and 518 intercellular VIGS assays due to some variations of TCV/GFP∆CP replication in different 519 plants.

520

### 521 **Bioinformatics Analysis of sRNA Sequences**

522 Illumina HighSeq 2000 sequencing produced 11 to 12 million reads per sRNA library. 523 The reads were cropped to remove adapter sequences and were aligned to the reference 524 sequences using Bowtie2 (Langmead and Salzberg, 2012; Ryabov et al., 2014). The 525 reference sequences included TCV/GFP∆CP, viral *TcvGFP* and *16cGFP* transgene 526 (Haseloff et al., 1997; Ruiz et al., 1998; Ryabov et al., 2004), *DCL1*, *DCL2*, *DCL3* and 527 *DCL4* gene sequences (Nakasugi et al., 2013) and the set of 50 tobacco microRNAs 528 identified in *Nicotiana* plants (Nakasugi et al., 2014; Pandey et al., 2008). SAMtools 529 pileup was used to produce the siRNA and miRNA coverage profiles. For correlation 530 analyses for the six small RNA libraries, we determined numbers of the miRNA hits 531 corresponding to the previously identified set of 50 *Nicotiana* miRNAs (Nakasugi et al., 532 2014; Pandey et al., 2008). All analyzed small RNA libraries contained similar 533 proportions of host-encoded miRNA reads (Supplemental Dataset S1; Supplemental 534 Dataset S2; Supplemental Dataset S3), indicating equivalence and direct comparability of 535 the sRNA datasets. Indeed outcomes of comparisons between normalized siRNAs 536 generated from target sequences against the total sRNA reads for all the libraries (per 10 537 million sRNA reads) are consistent with that the reads of siRNAs were directly 538 compared.

539

#### 540 **Statistical Analysis**

- 541 Normalized number of RNA silencing foci per leaf, sizes of RNA silencing foci,
- 542 "silencing cell-to-cell-spread index" (SCI) and qRT-PCR data between control and
- 543 various treatments were analysed by Student's *t*-Tests using an online programme
- 544 (http://www.physics.csbsju.edu/stats/t-test.html). It is worthwhile noting that
- 545 approximately 4% or more change in the silencing foci sizes is of statistical significance
- 546 due to the large numbers of samples (80-560) tested between wild-type controls and
- 547 RNAi lines (Fig. 1; Supplemental Table S2).

548

#### 549 **Supplemental Data**

- 550 The following materials are available in the online version of this article.
- 551 **Supplemental Text S1.** Parameters for Assessing Intra- and Intercellular VIGS.



- 583 We are grateful to David Baulcombe for his kind gift of the transgenic line *16cGFP* and
- 584 *RDR6i* seeds. The corresponding author thanks Dr Alison Tör for checking English 585 grammar and style throughout the manuscript.
- 586

# 587 **FIGURE LEGENDS**

### 588 **Figure 1. Different Roles of** *DCLs* **in the Cell-to-cell Spread of VIGS.** (A) Down-

- 589 regulation of *DCL* expression by RNAi. Young leaves were collected from *DCL* RNAi
- 590 plants at 7 days post-inoculation (dpi), and the level of *DCL* RNAs was analysed by qRT-
- 591 PCR. (B) Schematic of the intracellular RNA silencing trigger TCV/GFP∆CP. The T7
- 592 promoter, viral RNA-dependent RNA polymerases (P28, P88), movement proteins (P8
- 593 and P9) and GFP are indicated. (C-G) Restricted localization of TCV/GFP∆CP in single
- 594 epidermal cell of *Nb* (C), *DCL1i* (D) *DCL2Ai* (E), *DCL3Bi* (F) and *DCL4Ai* (G) plants.
- 595 (H-Q) Intercellular *GFP* silencing foci (dark patches indicated by red arrows).
- 596 Photographs of silencing foci on leaves of *16cGFP* (H); *GfpDCL1i* (I); *Gfp* (J);
- 597 *GfpDCL2Ai* (K) and *GfpDCL2Bi* (L); *GFPDCL3Ai* (M) and *GfpDCL3Bi* (N);
- 598 *GfpDCL4Ai* (O) and *GfpDCL4Bi* (P); and a triple-cross line *GfpDCL24i* (Q), were taken
- 599 under a fluorescent microscope at 7 dpi. Bar=500μm. (R) Normalized number of *GFP*
- 600 silencing foci per upper epidermis. Silencing foci were counted at 7 dpi from 3-21
- 601 different plant leaves inoculated with TCV/GFP∆CP. (S) and (T) Average size
- 602 (diameters, S) and percentage change (T) of silencing foci. 80-560 silencing foci on
- 603 different upper epidermises were randomly selected and measured. (U) SCI calculated as
- 604 percentage of number of silencing foci on lower epidermis out of the number of silencing
- 605 foci on upper epidermis. Student's *t*-tests were performed for qRT-PCR and silencing
- 606 data (mean  $\pm$  standard deviation) and P-values are indicated (asterisks).
- 607

# 608 **Figure 2. Regulation of** *DCL2* **Expression by** *DCL3* **and** *DCL4***.** (A-C) Effects of RNAi

- 609 of *DCL2* (A), *DCL3* (B) and *DCL4* (C) on *DCL* gene expression. Young leaf tissues were
- 610 collected at 6-8 leaf stage from four different plants of each transgenic line as indicated.
- 611 RNA transcripts were analysed by qRT-PCR. Four technical replicates for qRT-PCR
- 612 assays were performed on each cDNA of four biological duplicates ( $n = 4$ ; leaf tissues
- 613 from four different transgenic plants). Student's *t*-tests were performed for data (mean  $\pm$



- 645 inoculation. Blue and red bars represent siRNAs aligned to the sense and antisense
- 646 strands of the transgene *16cGFP* mRNA respectively.
- 647

# 648 **Figure 7. Size Profiles of TCV/GFP∆CP Viral siRNAs.** (A) *Gfp*. (B) *GfpDCL1i*. (C)

- 649 *GfpDCL2Ai*. (D) *GfpDCL3Bi*. (E) *GfpDCL4Ai*. The sRNA libraries were generated from
- 650 sRNA samples extracted from leaves with mock (left) or TCV/GFP∆CP (right)
- 651 inoculation. Blue and red bars represent siRNAs aligned to the sense and antisense
- 652 strands of TCV/GFP∆CP RNA, respectively.
- 653

654 **Figure 8. Cell- and Non-cell Autonomous VIGS in** *N. benthamiana***.** In incipient leaf 655 epidermal cells (*i.e.* individual cells initially infected by TCV/GFP∆CP), *DCL4* plays a 656 critical role in biogenesis of vsiRNAs, siRNA<sub>TCVGFP</sub> and transgene siRNA<sub>16cGFP</sub>. These 657 siRNAs are associated with cell-autonomous intracellular VIGS (Intra-VIGS) to inhibit 658 local virus infection. DCL4-processed siRNAs are unlikely involved in spread of VIGS 659 from leaf epidermal cell to adjacent cells because *DCL4* inhibits intercellular VIGS. In 660 the incipient cell, *DCL2* can also target and dice viral RNAs, *TcvGFP* and *16cGFP* 661 mRNA into siRNAs, but this activity is largely blocked by *DCL4* (**T** sign). In contrast, 662 the key functionality of *DCL2* is to trigger efficient intercellular VIGS (Inter-VIGS). This 663 is likely achieved through its activities to produce DCL2-processed/dependent siRNAs 664 (and/or their precursor long RNAs, highlighted red) in incipient cells and to perceive 665 these mobile signals for non-cell autonomous inter-VIGS in recipient epidermal and 666 mesophyll cells. Neither *DCL1* nor *DCL3* affects vsiRNA production, intra- and 667 intercellular VIGS. Thus *DCL4* and *DCL2* play major but distinct roles in cell- and non-668 cell autonomous VIGS that form a dual antiviral frontline in incipient and recipient cells. 669 *DCL4*, the primary defender for the cell-autonomous intracellular VIGS, can attack 670 viruses within the initially infected cells. However, if viruses break through this defence 671 frontline, non-cell autonomous intracellular VIGS can efficiently spread to nearby 672 recipient cells. This is due to loss of the negative control of intercellular VIGS mediated 673 by *DCL4*. Intercellular VIGS is dependent upon functional *DCL2* and DCL2- 674 processed/dependent siRNAs (and/or their precursor long RNAs), but it is negatively 675 controlled by *DCL4*. RNAi of *DCL4* results in fully functional *DCL2* that enhances cell-

- 676 to-cell spread of VIGS. The intercellular VIGS can then defend recipient cells from
- 677 further virus infection. Such a dual-defence strategy can compensate each other to give
- 678 host cells evolutionary advantage to battle against virus infection. This model is relevant
- 679 to virus-VIGS interaction at the intra-/intercellular level, rather than to systemic virus
- 680 infection. The potential spread of DCL2-processed/dependent siRNAs (and their
- 681 precursor long RNAs, highlighted red) to move from the incipient to recipient cell
- 682 through plasmodesmata is indicated with dashed arrows and cylinder signs.
- 683

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