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RNA interference-inducing hairpin RNAs in plants act through the viral defence pathway

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RNA interference (RNAi) is widely used to silence genes in plants and animals. It operates through the degradation of target mRNA by endonuclease complexes guided by approximately 21 nucleotide (nt) short interfering RNAs (siRNAs). A similar process regulates the expression of some developmental genes through approximately 21 nt microRNAs. Plants have four types of Dicer-like (DCL) enzyme, each producing small RNAs with different functions. Here, we show that DCL2, DCL3 and DCL4 in *Arabidopsis* process both replicating viral RNAs and RNAi-inducing hairpin RNAs (hpRNAs) into 22-, 24- and 21 nt siRNAs, respectively, and that loss of both DCL2 and DCL4 activities is required to negate RNAi and to release the plant's repression of viral replication. We also show that hpRNAs, similar to viral infection, can engender long-distance silencing signals and that hpRNA-induced silencing is suppressed by the expression of a virus-derived suppressor protein. These findings indicate that hpRNA-mediated RNAi in plants operates through the viral defence pathway.

Keywords: Dicer-like; siRNA; signal

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INTRODUCTION

Transforming plants with hairpin RNA (hpRNA) constructs (Fig 1A) has been used to discover or validate the functions of a steadily increasing number of genes. In several cases, the hpRNA-transformed plants phenocopy null mutants of the target genes (Wesley *et al*, 2001). The plant species *Arabidopsis thaliana* has four Dicer-like (DCL) proteins that produce differently sized small RNAs, which direct a suite of gene-silencing pathways. DCL1 produces microRNAs (Park *et al*, 2002), DCL2 generates both stress-related natural antisense transcript short interfering RNAs (siRNAs; Borsani *et al*, 2005) and siRNAs against at least two viruses (Xie *et al*, 2004; Akbergenov *et al*, 2006), DCL3 produces approximately 24 nt siRNAs that direct heterochromatin formation (Xie *et al*, 2004) and DCL4 generates both *trans*-acting siRNAs, which regulate some aspects of developmental timing, and siRNAs involved in RNA interference (RNAi; Dunoyer *et al*, 2005; Gasciolli *et al*, 2005; Xie *et al*, 2005). To obtain further details of the pathways involved in RNAi and virus defence, we examined the size and efficacy of small RNAs engendered by many RNAi-inducing hpRNAs, two distinct viruses and a viral satellite RNA (Sat) in different single and multiple *dcl*-mutant *Arabidopsis* backgrounds.

RESULTS AND DISCUSSION

Examining the siRNA profiles of more than 20 different hpRNA constructs in wild-type (WT) *Arabidopsis*, targeting either endogenes or transgenes, showed that the predominant size class is usually around 21 nt, with a smaller proportion around 24 nt RNAs (Fig 1B). To examine hpRNA-derived siRNAs in *dcl* mutants, an hpRNA construct (hpPDS), regulated by the *Arabidopsis* Rubisco small subunit (*SSU*) promoter, was made that targeted the phytoene desaturase (*PDS*) gene. Silencing *PDS* causes a photobleached phenotype in plants. The hpPDS construct was transformed into WT plants and into plants that were homozygous mutants for *dcl2*, *dcl3* or *dcl4-1*; it has already been shown that RNAi and siRNA production is uncompromised in the strongest DCL1 hypomorphic mutant available—*dcl1-9* (Finnegan *et al*, 2003). The primary WT and *dcl2* transformants showed similar degrees of photobleaching, and *dcl3* transformants showed extreme photobleaching (Fig 1C). This shows that neither DCL2 nor DCL3 activity is required for RNAi and suggests that the absence of DCL3 activity enhances silencing. Interestingly, the *ago4* mutant background has also been reported to enhance hpRNA-mediated silencing (Zilberman *et al*, 2004). Both DCL3

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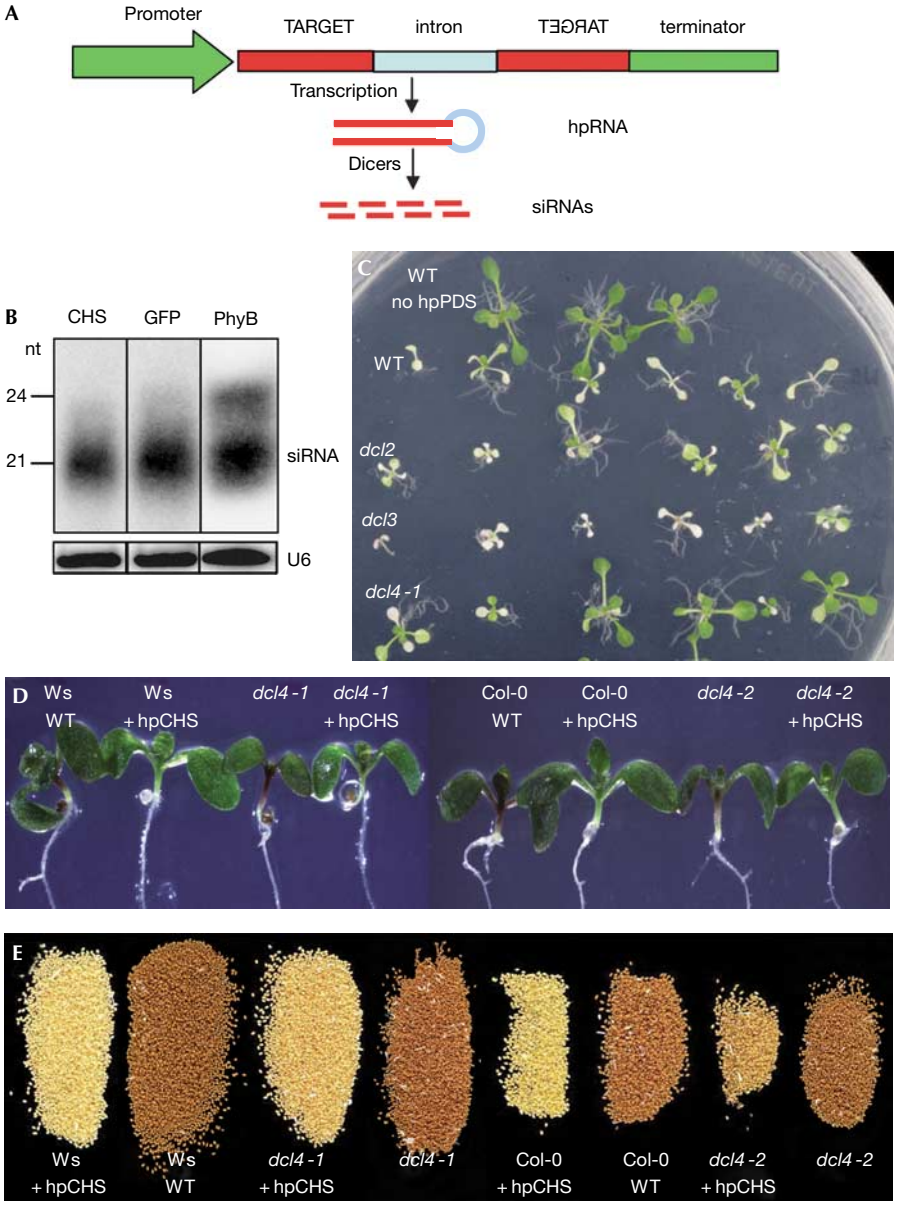

Fig 1 | Involvement of Dicer-like proteins in hairpin RNA-mediated silencing. (**A**) Schematic representation of hairpin RNA (hpRNA) constructs used in plants and their processing to RNA-interference-directing short interfering RNAs (siRNAs). (**B**) Northern blot analysis of siRNAs from *Arabidopsis* plants containing different hairpin transgenes: CHS, chalcone synthase; GFP, green fluorescent protein; PhyB, phytochrome B. The lower panel shows U6 transcript as a loading control. (**C**) Photobleaching in 2-week-old wild-type Col-0 (WT) and *dcl2*, *dcl3* and *dcl4-1* single mutants transformed with an hpPDS transgene and control untransformed WT plants. The plants, arranged in rows, are independent primary transformants. Milder silencing is obvious in *dcl4-1*. (**D**,**E**) Chalcone synthase silencing in young seedlings (**D**) and seeds (**E**) from left to right in pairs—transformed with hpCHS and untransformed—Ws, *dcl4-1* mutant, Col-0, and *dcl4-2* mutant.

and AGO4 are involved in siRNA-directed chromatin condensation; so it is possible that the silencing enhancement is due to a reduction in transcriptional repression of the hpRNA transgene. The mild silencing in *dcl4-1* suggests that DCL4 activity is important, but not essential, for RNAi. To test this further, *dcl4-1* and a different line (*dcl4-2*) were transformed with an hpRNA construct targeting the chalcone synthase (*CHS*) gene. CHS is required for anthocyanin production, and silencing the gene reduces the production of red/brown pigment in the hypocotyls of young seedlings and in the seed coat. Two out of ten *dcl4-1* and two out of ten *dcl4-2* lines, independently transformed with hpCHS, had green hypocotyls and yielded pale seeds (Fig 1D,E), affirming that DCL4 activity is not essential for RNAi. Therefore, we investigated whether DCL2 was processing hpRNA into RNAi-mediating siRNAs in the absence of DCL4.

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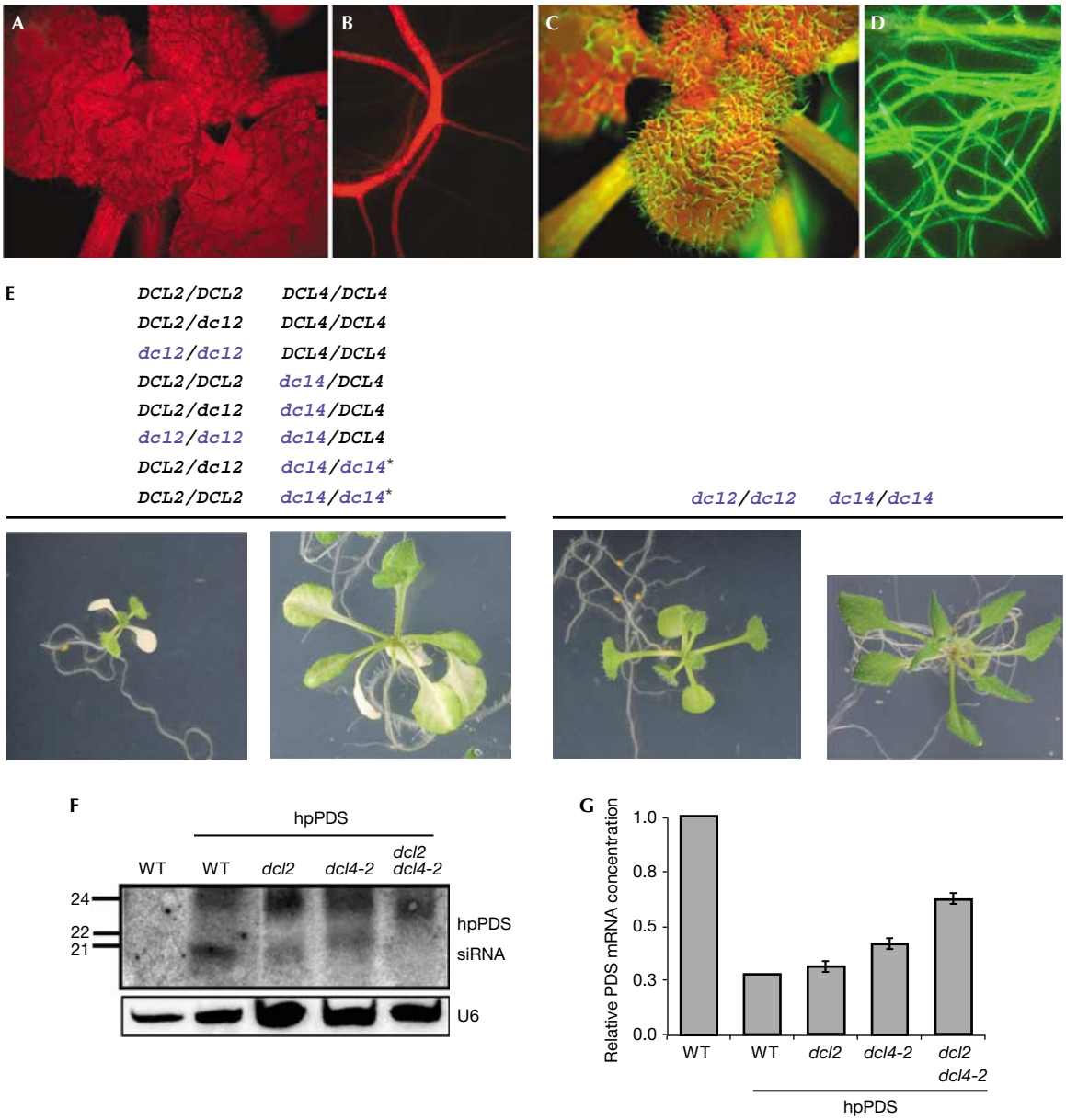

Fig 2 | *DCL2* and *DCL4* are both involved in hairpin RNA-mediated silencing. (**A**,**B**) Green fluorescent protein (GFP) silencing in the shoot (**A**) and root (**B**) of a *dcl4-1* mutant plant carrying a GFP–hpGFP construct. (**C**,**D**) GFP–expression in the shoot (**C**) and root (**D**) of a *dcl2 dcl4-1* double-mutant plant carrying a GFP–hpGFP construct. (**E**) Genotypes of hpPDS/*dcl2 dcl4* double mutants showing strong (left) and no (right) photobleaching in 2- and 4-week-old segregating seedlings. Asterisks indicate genotypes with milder phenotypes. (**F**) Northern blot analysis of 21–24 nt short interfering RNAs derived from the hpPDS in *dcl2*, *dcl4* and *dcl2 dcl4* mutants after segregation. (**G**) Real-time quantitative PCR analysis of absolute *PDS* transcript levels from 4-week-old wild-type (WT) and *dcl* mutant plants carrying an hpPDS transgene. DCL, Dicer-like; hp, hairpin; PDS, phytoene desaturase.

A construct containing a green fluorescent protein (GFP) gene and an hpRNA transgene against GFP (hpGFP) was transformed into *dcl4-1* and *dcl4-1 dcl2* lines. No primary hpGFP/*dcl4-1* transformants showed any GFP expression (Fig 2A,B), but five primary hpGFP/*dcl4-1 dcl2* transformants expressed GFP (Fig 2C,D). This suggested that RNAi can operate in the absence of DCL4, but not in the absence of both DCL4 and DCL2. To examine this further, a crossing strategy was undertaken by using a fertile hpPDS/*dcl2* line, showing moderate photobleaching, from the experiment depicted in Fig 1C. Strongly silenced lines could not be used because they were impaired by photobleaching to such an extent that they died before flowering. The selected hpPDS/*dcl2* line was crossed with *dcl4-2* to produce an F_1 double-heterozygous plant that had also inherited hpPDS. Self-pollinated F_2 progeny from this plant were germinated on media selective for inheritance of the hpPDS construct and monitored for symptoms of photobleaching. Most of the seedlings showed photobleaching, but a few were unbleached (Fig 2E). Genotyping the unbleached

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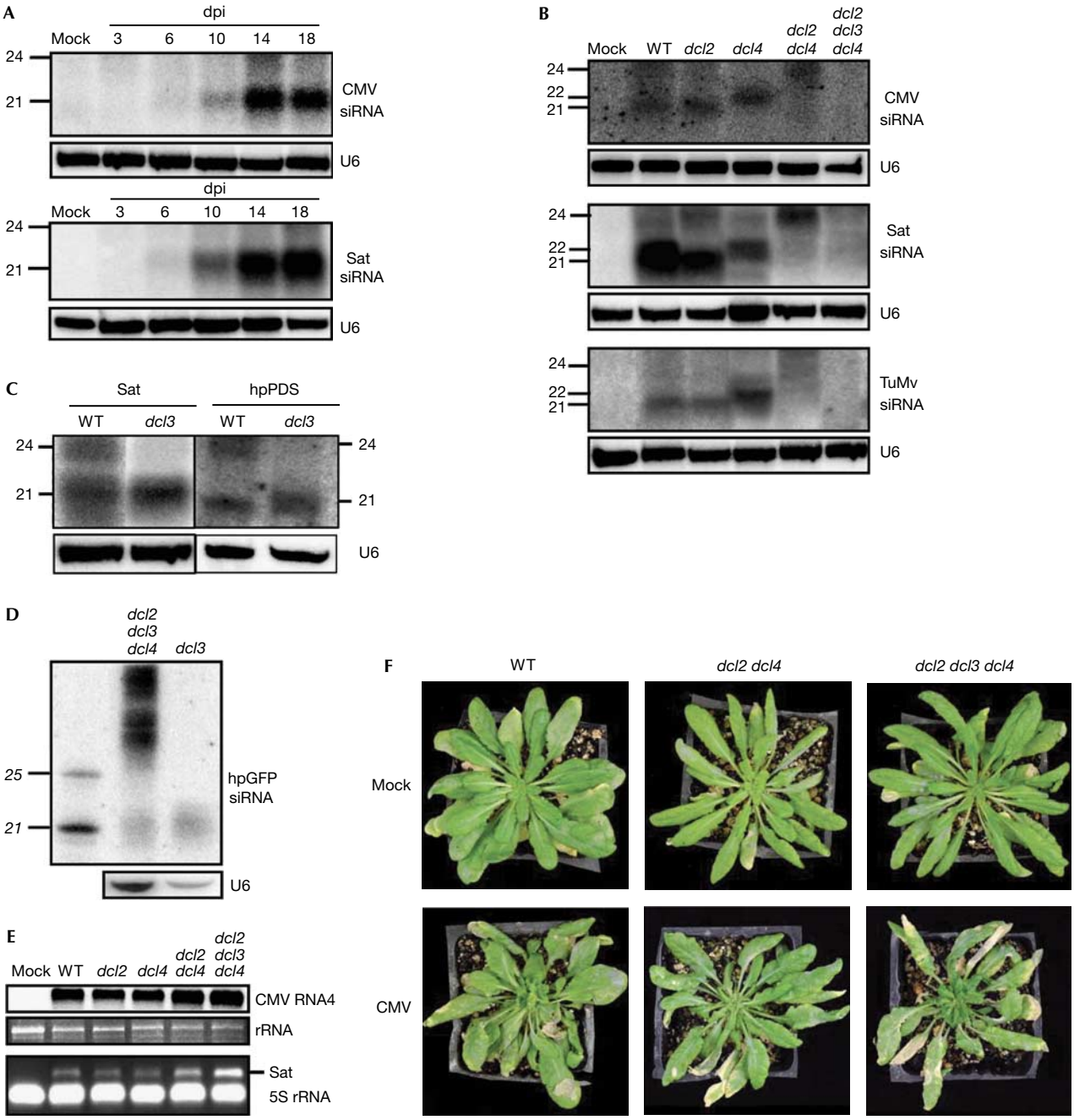

Fig 3 | *DCL2*, *DCL3* and *DCL4* are involved in the viral defence pathway. (**A**) Time-course analysis of short interfering RNA (siRNA) accumulation in wild-type (WT) *Arabidopsis* Col-0 plants infected with cucumber mosaic virus (CMV; upper panel) and CMV + satellite (Sat; lower panel), 3–18 days post-inoculation (dpi). (**B**) Northern blot analysis of 21–24 nt siRNAs derived from WT and *dcl* mutant plants infected with CMV (upper panel), CMV + Sat (middle panel) and tobacco mosaic virus (TuMV; lower panel) at 18 dpi. (**C**) Detection of siRNAs derived from CMV Sat (left) and from an hpPDS transgene (right) in WT and *dcl3* plants. (**D**) Detection of small RNAs derived from hpGFP RNA in *dcl3* and *dcl2 dcl3 dcl4-2* mutants. (**E**) Detection of CMV RNA4 by northern blot analysis of total RNA from WT and *dcl* mutant plants (upper panel). Ribosomal RNA was used as a loading control (middle panel). The lower panel shows accumulation of CMV Sat RNA in an ethidium-bromide-stained gel after purification of the small RNA fraction. Higher accumulation of viral and Sat transcripts is evident in the *dcl2 dcl4-2* and *dcl2 dcl3 dcl4-2* mutants (upper and lower panels). (**F**) Phenotypes of WT Col-0 (left panels), *dcl2 dcl4-2* (middle panels) and *dcl2 dcl3 dcl4-2* (right panels) mutants, mock inoculated or infected with CMV, at 18 dpi. (**A–D**) U6 RNA was used as a loading control. DCL, Dicer-like; hpGFP, hairpin green fluorescent protein; hpPDS, hairpin phytoene desaturase.

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seedlings showed that they were double-homozygous *dcl2 dcl4-2*. Seedlings with the other possible combinations of genotype showed a degree of photobleaching similar to that of the parental hpPDS/*dcl2* line, except for a few that had slightly less severe photobleaching and were homozygous *dcl4-2* in combination with either heterozygous *dcl2* or *DCL2* WT. The hpPDS siRNA profiles in the different genotypes showed that there were 21 and 24 nt siRNAs in both WT and *dcl2*, 22 and 24 nt siRNAs in *dcl4-2*, and only 24 nt siRNAs in *dcl2 dcl4-2* (Fig 2F). These results indicated that (i) the 24 nt siRNAs have no role in directing mRNA degradation, (ii) 21 nt siRNAs are produced by DCL4 and are the principal components directing the messenger RNA degradation, and (iii) DCL2 (especially in the absence of DCL4) produces 22 nt siRNAs that can also direct mRNA degradation. This was largely confirmed by analysis of the *PDS* mRNA levels (Fig 2G). Compared with untransformed WT, the *PDS* mRNA levels in hpPDS/WT, hpPDS/*dcl2*, hpPDS/*dcl4-2* and hpPDS/*dcl2 dcl4-2* were 28, 31, 42 and 61%, respectively. These data suggest that in the presence of DCL4, DCL2 directs a small percentage of the RNAi activity, but when DCL4 is absent, DCL2 directs RNAi that is only 10–20% less efficient than DCL4 alone. The incomplete restoration of the *PDS* mRNA in hpPDS/*dcl2 dcl4-2* to WT levels indicates that the DCL3-produced 24 nt siRNAs reduce *PDS* mRNA, but to a level insufficient to cause photobleaching. As 24 nt siRNAs are known to direct chromatin modification, it is possible that they are reducing *PDS* transcription. Alternatively, they might be directing some *PDS* mRNA cleavage.

To examine the roles of the differently sized siRNAs in defending plants against viruses, the range of *dcl* mutants was challenged with turnip mosaic virus (TuMV) and cucumber mosaic virus (CMV), with or without its Sat. About 18 days post-inoculation (dpi), siRNAs derived from CMV or Sat were readily detectable in WT *Arabidopsis* plants (Fig 3A). Analysing the *dcl* mutants at 18 dpi with CMV, CMV + Sat or TuMV (Fig 3B) showed essentially the same siRNA/*dcl* mutant profiles as those obtained for the hpPDS/*dcl* mutants. There were 21 and 24 nt siRNAs in both WT and *dcl2*, 22 and 24 nt siRNAs in *dcl4-2* and only 24 nt siRNAs found in *dcl2 dcl4-2*. In *dcl3* plants infected with CMV + Sat or transformed with the hpPDS construct (Fig 3C), 21 nt siRNAs were abundant, but the production of 24 nt siRNAs was abolished. These results indicated that, in plants, hpRNAs are processed into siRNAs and used to target RNA degradation by the same enzymes and cofactors used to recognize and restrain viruses.

A *dcl2 dcl3 dcl4* triple mutant line was challenged with CMV, CMV + Sat or TuMV and also transformed with an hpGFP construct. No siRNAs were detectable in this triple mutant challenged with the different viruses (Fig 3B), whereas a low level of 21 nt siRNAs and RNAs greater than 30 nt were detected in the line transformed with the hpRNA construct (Fig 3D). A similar small RNA profile was recently detected from an endogenous dispersed repeat in *dcl2 dcl3 dcl4* (Henderson *et al*, 2006). Presumably, these small RNAs are produced by DCL1 and similarly produced small RNAs might also have a role in the incomplete restoration of the *PDS* mRNA to WT levels in hpPDS/*dcl2 dcl4* (Fig 2G). The steady-state levels of CMV and Sat genomic RNAs and the severity of their symptoms were higher in *dcl2 dcl4* and *dcl2 dcl3 dcl4* than in WT plants (Fig 3E,F; supplementary Fig S2 online). Interestingly, the Sat genomic RNA levels were even higher in the triple mutant than in the double mutant, suggesting that DCL3 has a role in restricting the replication and/or accumulation of the satellite, and is consistent with the incomplete return of *PDS* mRNA to WT levels in the hpPDS-*dcl2 dcl4* plants. However, it is in contrast with the increased, rather than decreased, silencing observed for the hpPDS in *dcl3* mutants. These dichotomous consequences might be explained by DCL3 cleaving the transient double-stranded replicative form of the Sat to reduce directly its steady-state level, whereas DCL3 compromises RNAi by cleaving the stems of hpRNA into less-effective RNAi-directing 24 nt siRNAs that would otherwise have been processed by DCL4 and DCL2 into the more active 21 and 22 nt siRNAs, respectively.

If hpRNAs are processed in the same manner as double-stranded RNA from an invading virus, they might also evoke other virus-like characteristics. Virus-infected cells generate and transmit long-distance signals to uninfected cells that trigger a silencing-like response that defends against virus spread (Sonoda & Nishiguchi, 2000; Voinnet, 2005). Also, viruses contain suppressor proteins that suppress the viral defence response (Voinnet, 2005). Therefore, we carried out experiments to test whether hpRNAs are processed to produce such a signal, and whether RNAi directed by hpRNAs could be prevented by the transgenic expression of a viral suppressor protein, HC-Pro (Mallory *et al*, 2001). Scions from a tobacco plant expressing a β-GLUCURONIDASE (GUS) reporter gene were grafted onto rootstocks from plants transformed with an anti-GUS hpRNA construct, and scions from *Arabidopsis* plants expressing GFP were grafted onto rootstocks transformed with an anti-GFP hpRNA construct. In both systems, the reporter gene in the newly developing tissues of the scion was silenced (Fig 4A–F). Tobacco plants containing an anti-potato virus Y construct (hpPVY) and sibling plants also expressing HC-Pro were analysed for their response to inoculation with PVY. The plants containing hpPVY were protected against PVY, whereas plants containing the same construct in the HC-Pro background were susceptible to the virus (Fig 4G). Both sets of results indicate further that hpRNAs are processed by the viral defence pathway.

Before the complexity and diversity of microRNAs, Dicers and silencing-related pathways were understood, it was proposed that hpRNAs operate by directing an intrinsic viral defence system against the target gene's mRNA (Waterhouse *et al*, 2001). Our results indicate that this is indeed the case.

METHODS

Plant material, transformation and imaging. *Arabidopsis* mutants *dcl2*, *dcl3* and *dcl4-2* are in the Col-0 ecotype and were identified in the Salk T-DNA collection (Salk_064627 and Salk_005512) and GABI-Kat collection (GABI160G05), respectively. The *dcl4-1* mutant, identified in the INRA T-DNA collection (FLAG_330A04), is in the Ws ecotype. Although these lines were independently acquired, propagated and screened for homozygosity, derivatives from the same original insertion lines have been described elsewhere (Xie *et al*, 2004, 2005; Gasciolli *et al*, 2005). The double mutants *dcl2 dcl4-1* and *dcl2 dcl4-2* and the triple mutant *dcl2 dcl3 dcl4-2* were generated by standard genetic crosses. Homozygous genotypes were validated by PCR analyses after two generations. The tobacco (*Nicotiana tabacum*) HC-Pro/hpPVY plant line is from a cross between a line carrying a PVY hpRNA

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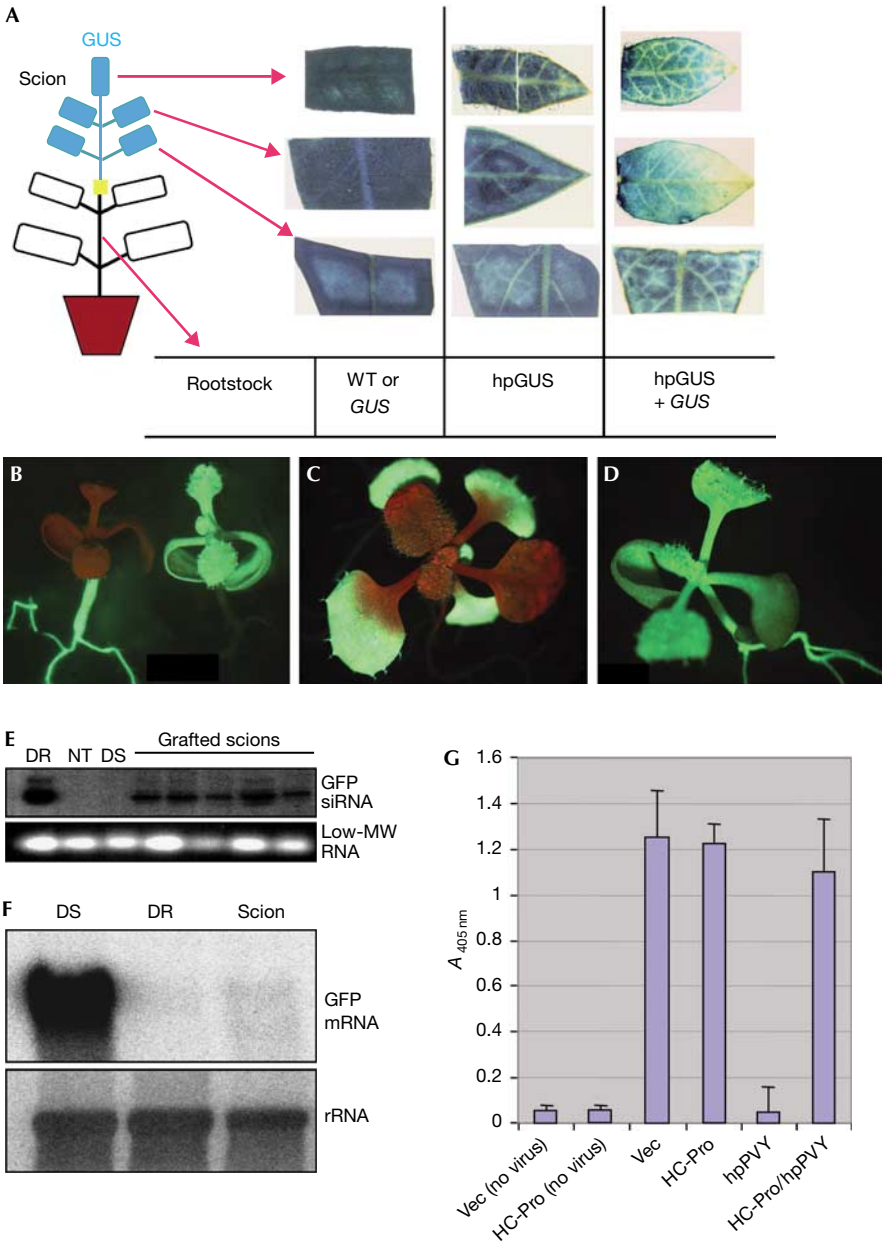

Fig 4 | A systemic silencing signal is triggered by hairpin transgenes. (**A**) Diagram of a grafting experiment, showing the effect of scions expressing a *GUS* reporter gene grafted onto a wild-type (WT) tobacco rootstock (left panel), onto a rootstock expressing a hairpin GUS (hpGUS) construct (middle panel) and onto a rootstock containing a *GUS* gene silenced by an hpGUS construct (right panel). Newly developed leaves of the scion show *GUS* silencing mainly in the vascular tissue of the hpGUS graft and a more pronounced silencing in the hpGUS + *GUS* graft. (**B**) Control experiment, showing reciprocal grafts of a WT *Arabidopsis* Col-0 scion grafted onto a rootstock expressing green fluorescent protein (GFP) reporter gene (left) and a GFP-expressing scion grafted onto a WT rootstock (right). GFP expression is not affected in either of the grafts. (**C**) GFP-expressing scion grafted onto a rootstock expressing a GFP–hpGFP construct. The newly developed leaves of the scion, 3 weeks after grafting, show GFP silencing (red autofluorescence) owing to the spread of the systemic silencing signal generated by the hpGFP in the rootstock. (**D**) GFP-expressing scion parent line 3 weeks after germination. (**E**) Northern blot analysis (upper panel) of 21–24 nt short interfering RNAs present in the donor GFP-expressing scion (DS), donor hpGFP rootstock (DR) and five individual scions (grafted scions) 3 weeks after grafting. (**F**) Northern blot analysis (upper panel) of GFP messenger RNA levels in DS, DR and five pooled scions (scion) 3 weeks after grafting. Ribosomal RNA was used as a loading control. (**G**) Viral levels in tobacco plants carrying an hpPVY transgene (hpPVY), an HC-Pro silencing suppressor transgene (HC-Pro), both transgenes (HC-Pro/hpPVY) or the empty vector (Vec). Eight plants of each line were inoculated with PVY-D strain. Virus levels were measured by enzyme-linked immunosorbent assay at 3 weeks post-inoculation. The columns represent median values, and error bars are derived from standard error values. GUS, β-GLUCURONIDASE; PVY, potato virus Y.

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construct (Smith *et al*, 2000) and a line expressing HC-Pro (kindly provided by Dr Vicki Vance, University of South Carolina, USA). The GUS silenced plants, containing the *GUS* transgene and a GUS hairpin construct, have been described previously (Wesley *et al*, 2001).

Arabidopsis transformations were by the floral dip method (Clough & Bent, 1998). It was not possible to use antibiotic selection in the transformation of *dcl4-1* and *dcl4-1 dcl2* with hpGFP, because these FLAG-db T-DNA insertion lines contained both BASTA and kanamycin resistance. However, the screen was for inactivation of silencing that would allow expression of GFP. Therefore, 2,000 seeds from dipped plants were plated without selection and screened for GFP expression. Two thousand seeds from WT Ws plants, dipped with the hpGFP construct in the same experiment, yielded 16 transformants. Images of GFP-expressing plants were taken under blue light by using a Leica MZ FLIII dissecting microscope coupled to a Zeiss AxioCam HRc camera.

Hairpin transgenes. HpRNA transgenes, containing spliceable introns, were constructed in a variety of related vector backbones. With the exception of hpPDS, all are expressed from the cauliflower mosaic virus 35S promoter. The chalcone synthase hairpin construct contains 741 nt of the *CHS* coding sequence in pHannibal (Wesley *et al*, 2001). The phytochrome B hpRNA construct was generated in pHellsgate8 (Helliwell *et al*, 2002) by using a 298-bp coding region fragment. The PDS hairpin construct contains 432 nt of the *PDS* coding sequence in pWatergate (http://www.pi.csiro.au/techlicensing_biol/GeneSilencingVectors.htm). A GFP expression vector was constructed by cloning full-length GFP behind p35S into a binary vector derived from pNB96, which was obtained from Dr Hong-Gil Nam (POSTECH, Pohang, Republic of Korea). The hpGFP vector was derived from the expression vector by the addition of a pHannibal-derived hairpin cassette with hairpin arms containing nucleotides 9–400 of the *GFP* coding sequence.

Grafting experiments. *Arabidopsis* butt grafting was carried out according to Turnbull *et al* (2002). Tobacco wedge grafting was conducted similar to that described by Palauqui *et al* (1997), but with modifications described by Crete *et al* (2001). Tobacco grafts were tested for GUS activity by using the histochemical stain X-glucuronide, as described by Jefferson *et al* (1987).

Virus infection assays. *Arabidopsis* plants were grown *in vitro* for 2 weeks, then transferred to soil and grown for 3 weeks under short-day conditions before virus inoculation. Plants were mock-inoculated with phosphate buffer or inoculated with sap extracts (diluted in 0.05 M phosphate buffer (pH 7.0)) from plants infected with CMV, CMV + Y-Satellite or TuMV. Plant material was collected at 18 dpi for RNA extraction. Tobacco plants were grown in standard greenhouse conditions for 4 weeks before inoculation with PVY strain D and collected at 14 dpi.

RNA preparation and blot assays. Total RNA (5 μg) from aerial parts of virus-infected plants was prepared by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Small RNAs from whole plants were extracted according to Han & Grierson (2002) and 20 μg samples were separated on 15% polyacrylamide gels. RNAs were transferred to Hybond N+ membrane (Amersham Bioscience, Piscataway, NJ, USA) and blots were hybridized with [^{32}P]UTP-labelled riboprobes obtained by *in vitro* transcription from the respective sequences cloned into pGEM plasmids (Promega, Madison, WI, USA). Pre-hybridization and hybridization of blots were carried out at 38 °C in 125 mM Na_2HPO_4 (pH 7.2), 250 mM NaCl, 7% SDS and 50% formamide. Membranes were washed twice with 2 × SSC and 0.2% SDS for 30 min at 38 °C and imaged by using a Fujifilm FLA-5000 phosphorimager. Synthetic RNA oligonucleotides were used as size markers.

Real-time quantitative reverse transcription–PCR. The *PDS* primer pair, forward 5′-CAAACTGTGAACCATGTCG-3′ and reverse 5′-AGCAGAATTTGCCAGAGAGG-3′, was designed by using Primer3 (Rozen & Skaletsky, 2000). Real-time PCR reactions were carried out as described previously (Millar *et al*, 2006). *PDS* transcript levels in WT and *dcl* mutants were quantified by using 5 μg samples of total RNA, reverse-transcribed into complementary DNA. PCR cycles were as follows: one cycle of 94 °C for 5 min; 45 cycles of 94 °C for 15 s denaturation, 60 °C for 15 s annealing and one cycle of 72 °C for 30 s extension. Results were normalized against cyclophilin and quantification of each cDNA synthesis was carried out in triplicate.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

Note added in the proof. During the review process, work by Deleris *et al* (doi:10.1126/science.1128214; 2006) and Bouche *et al* (doi:10.1038/sj.emboj.7601217; 2006) was published, which similarly shows that DCL4 and DCL2 are involved in the production of viral siRNAs.

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