



*Original Article*

# Effect of dicer-like proteins2 and 4 and RNA-dependent RNA polymerase1 as RNA silencing components on cyclic mosaic symptom development in tobacco infected with the *Cucumber mosaic virus*

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Received 16 January 2012; Accepted 13 May 2013

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## Abstract

The *Nicotiana tabacum* genome contains four Dicer-like proteins (DCLs) and six RNA-dependent RNA polymerase (RDR) homologues involved in the RNA silencing mechanism employed against viral infection. DCL1 synthesizes 18-21 nt-long microRNA, whereas DCL2, DCL3 and DCL4 produce 22 nt, 24 nt and 21 nt-long siRNA, respectively, in the RNA silencing process. This study aimed to clarify which components among these are involved in changes in the amount of virus and the development of symptoms in *Cucumber mosaic virus* (CMV)-infected tobacco. Infected transgenic tobacco lines with a single down-regulation of DCL2, DCL4, RDR1 or a double down-regulation of both DCL2 and 4 were analyzed. The amounts of viral RNA in young developing leaves in transgenic tobacco lines were examined by Northern blot analysis. Most transgenic plants inoculated with CMV Pepo, a virulent strain, exhibited cyclic mosaic symptoms. The amount of viral RNA in single down-regulated lines varied based on leaf position in a similar manner to that noted in non-transgenic tobacco, while that of the double down-regulated line did not. Furthermore, the expression of RNA-silencing-related genes during high and low CMV infection did not differ among the transgenic plants. These results suggested that (i) changes in the amounts of the virus in the developing leaves of all the single down-regulated lines were associated with cyclic symptom expression in fully expanded leaves, and (ii) the lower expression of DCL2, DCL4 and RDR1 may be sufficient to establish cyclic symptom development.

**Keywords:** dicer-like proteins, RNA-dependent RNA polymerase, RNA silencing, *Cucumber mosaic virus*, mosaic symptom

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## 1. Introduction

Virus infection in a plant host activates a molecular defense mechanism known as RNA silencing or RNA interference (RNAi) which causes the degradation of viral RNA. The antiviral role of RNA silencing has been extensively studied in plants (Voinnet, 2005; Ding and Voinnet, 2007; Marques and Carthew, 2007). It is induced by double-stranded viral RNA (dsRNA) or by specific single-stranded RNA (ssRNA) structures. The essential components of RNA silencing are RNA-dependent RNA polymerases (RDRs) and

dicer-like ribonuclease proteins (DCLs). The RDRs synthesize complementary RNA by using viral RNA as a template. The DCL active in plant antiviral silencing is an endoribonuclease in the RNase III family that cleaves dsRNA into about 21-24 nucleotides (nt)-long fragments known as small interfering RNA (siRNA), which direct sequence-specific cleavage of viral RNA by Argonaute proteins (Blevins *et al.*, 2006; Wang *et al.*, 2011).

In plants, host-encoded RDR may use viral RNA templates to produce dsRNAs that serve as the substrate for the formation of siRNA by DCLs (Wang *et al.*, 2010). As an example, the genome of the plant *Arabidopsis thaliana* encodes four DCL enzymes, six RDRs, and other components of the silencing machinery found in plants. It has been dis-

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covered that systemic RNA silencing in *Arabidopsis* requires RDR1 and RDR6 for the amplification of CMV-derived small RNA (Wang *et al.*, 2010; 2011). Although RNA viruses are mainly targeted by DCL4 and DCL2 in antiviral RNA silencing (Xie *et al.*, 2004; Deleris *et al.*, 2006; Bouché *et al.*, 2006; Diaz-Pendon *et al.*, 2007), DCL2 can substitute for DCL4 to some extent in *dcl4* mutants (Deleris *et al.*, 2006; Diaz-Pendon *et al.*, 2007). In *Arabidopsis*, DCL2, 3, and 4 are predominantly responsible for cleavage against positive-sense RNA viruses, including the *Cucumber mosaic cucumovirus* (CMV), *Oilseed rape mosaic tobamovirus*, and the *Turnip crinkle carmovirus*, (TCV) (Blevins *et al.*, 2006; Bouché *et al.*, 2006; Deleris *et al.*, 2006; Moissiard and Voinnet, 2006; Diaz-Pendon *et al.*, 2007; Qu *et al.*, 2008).

RNA silencing is likely to be involved in disease phenotypes such as the mosaic symptom developed by plants infected by certain viruses. To counteract this defense mechanism, many viruses have evolved suppressors for RNA silencing (Roth *et al.*, 2004; Lewsey *et al.*, 2009). In our previous study, we found that the 2b protein of CMV Pepo strain, which acts as a silencing suppressor, determines the virulence by facilitating the distribution of CMV in meristem tissues and leaf primordia, probably by inhibiting the antiviral RNA silencing, and thus inducing cyclic mosaic symptoms in tobacco plants (Sunpapao *et al.*, 2009, Sunpapao *et al.*, 2011). Indeed, Fukuzawa *et al.* (2010) showed that in transgenic *Nicotiana benthamiana* that the HC-Pro, a potyvirus RNA silencing suppressor, in was constitutively expressed canceled cyclic symptom expression upon CMV infection. However, the role of RNA silencing components in the development of cyclic mosaic symptoms has not yet been clarified. It is hypothesized that DCLs and RDRs may participate in the development of cyclic mosaic symptoms by preventing the virus spreading to the meristem and other developing tissues.

To investigate which RNA silencing components are involved in varying the amounts of the virus infection by CMV and the development of mosaic cyclic symptoms, transgenic plants in which expression of *DCL2*, *DCL4* and *RDR1* mRNA had been down-regulated were used. Following the CMV infection, the uninoculated upper leaves of tobacco plants appear alternately mosaic and mottle, then symptomless, and finally again become mosaic (Figure 1A): this phenomenon is typified as cyclic mosaic symptom expression (Ohki *et al.*, 1990; Gal-On *et al.*, 1996; Kaplan *et al.*, 1997). We found that the single down-regulated transgenic tobacco plants had similar cyclic mosaic phenotypes as the non-transgenic plants, and symptom severity was associated with virus concentrations.

## 2. Materials and Methods

### 2.1 Plant materials, CMV and inoculation

Tobacco plants (*N. tabacum* cv. Samsun, Hirai *et al.*, 2008; Hirai, 2009) used in this study were provided by

Dr Meshi of the National Institute of Agrobiological Sciences, Tsukuba, Japan. The expression of *DCL2*, *DCL4*, or *RDR1* mRNA in these plants was down-regulated by inverted-repeat constructs: this generated the tobacco lines *dcl2i*, *dcl4i*, *rdr1i*, and double mutant *dcl24i* in this study. The Pepo strain of CMV was originally obtained from *Cucurbita pepo* (Osaki *et al.*, 1973). Five to seven-leaf stage tobacco plants were inoculated with a purified Pepo. The largest leaf of each plant was mechanically inoculated with the virus at 25 µg/ml and designated as leaf position 0 (L0). The leaves above the inoculated leaf were sequentially numbered. The inoculated plants were grown in a room, and maintained in conditions similar to those in a greenhouse.

### 2.2 RNA analysis

Total RNA was extracted from the tissues of young developing leaves (YDLs), 1-2 cm in size, using TriPure Isolation Reagent (Roche Diagnostics) according to the manufacturer's instruction. For the detection of the CMV genomic RNAs, 1 µg of total RNA was loaded onto a 1.5% denaturing agarose gel. The procedures for blotting onto Hybond-N+ membrane (GE Healthcare Biosciences), hybridization with DIG-labeled RNA probe complementary to the conserved 3'-UTR sequence, and detection of signals, have been previously described by Kobori *et al.* (2002).

### 2.3 Semi-quantitative RT-PCR

The reduction of the RNA silencing components in the genes was determined by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR), adapted from Serazin-Leroy *et al.* (1998). RT was performed in 20 µl reaction mixtures containing 1 µg total RNA template, 2.5 mM dNTPs, 5X RT buffer, RNase inhibitor, 50 units of ReverTra Ace (TOYOBO), and 100 mM dT15 primers. RT reaction was

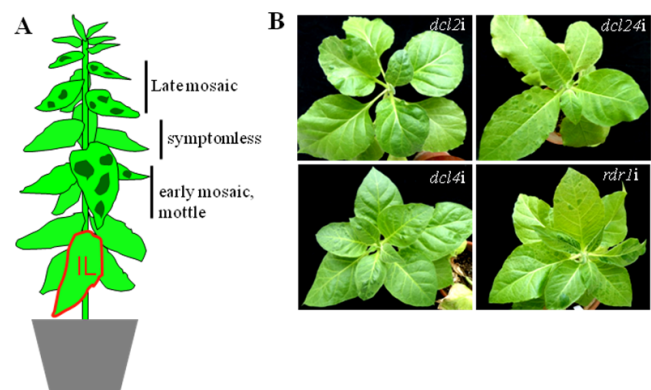


Figure 1. Schematic representation of cyclic mosaic symptoms in tobacco plant (A): symptomless leaves are interspersed among mottle or mosaic leaves along the stem of each individual plant (IL = inoculated leaf). Symptoms on *dcl2i*, *dcl4i*, *rdr1i* and *dcl24i* on CMV inoculated transgenic lines (B).

performed using the Gene Amp PCR system 9700 thermal cycler (Perkin Elmer). The RT reaction was carried out at 30°C for 10 minutes, at 42°C for 60 minutes, and at 95°C for 5 minutes. The cDNA product was diluted with distilled water (DW).

PCR was performed in 10 µl reaction mixtures containing 10X EX-taq buffer, 2.5 mM dNTPs, 0.5 units of Ex-taq (TAKARA), 5 µM forward and reverse primers corresponding to *DCL2*, *DCL4* and *RDR1* (Table 1), and 3 µl of cDNA product. After pre-heating at 96°C for 5 minutes, the PCR was carried out for 28, 30 and 32 cycles of respectively 96°C denaturation for 30 seconds, 60°C annealing for 30 seconds, and 72°C extension for 15 seconds. The ubiquitin (*UBI*) housekeeping gene fragment was used as the internal control via the specific primers UBI-1 and UBI-2 (Table 1). The PCR products were separated by 1% agarose gel electrophoresis and were stained with ethidium bromide.

### 3. Results

#### 3.1 mRNA expressions of the down-regulated RNA silencing components in transgenic lines

Low expression of mRNAs of RNA silencing components in the transgenic plants was confirmed by semi-quantitative RT-PCR with specific primers corresponding to *DCL2*, *DCL4* and *RDR1* (Table 1, Hirai, 2009). Amounts of mRNAs in transgenic plant lines were compared with those in the non-transgenic plant. The quantity of ubiquitin mRNA was used as an internal control. The mRNA amount of each *DCL2*, *DCL4*, or *RDR1* was low in corresponding transgenic plant lines, e.g. only *DCL2* amount was low in *dcl2i* tobacco while both *DCL2* and *DCL4* were low in *dcl24i* tobacco (Figure 2).

#### 3.2 Symptom patterns

To analyze the effects of down-regulation of RNA silencing components on the cyclic mosaic symptom

Table 1. Primers used for cDNA synthesis and PCR amplification of DNA fragments used to analyze DCLs and RDRs mRNA.

Primers	Primer Sequences (5' to 3')
DCL2-1	TGTTCTGGTGGCTCAGCAAGG
DCL2-2	CCATGTAGCAGCATCCCAATAATCG
DCL4-1	GGCTTGAAGTGCTTGGTGATG
DCL4-2	TTCTGACTGCTATCGTGACGG
RDR1-1	GGTGTTGCTGTGAGGTGTTTTG
RDR1-2	ACCAAGCCGAAGCCTTTGG
Ubiquitin-1	TCCAGGACAAGGAGGGTATCC
Ubiquitin-2	TAGTCAGCCAAGGTCCTTCCAT

Primers "1" are forward, primers "2" are reverse.

development, twenty plants of each transgenic tobacco line (*dcl2i*, *dcl4i*, *dcl24i*, or *rdr1i*) were inoculated with CMV, and the progress of the symptoms was observed. Mosaic symptom of transgenic lines started to appear on the YDLs in infected plants 7 days after inoculation (dpi). All transgenic lines developed both mottle and mosaic symptoms (CMV cyclic mosaic symptom type) at 30 dpi, along the stems of individual plants (Figure 1B and Table 2): there were some symptomless leaves interspersed among the affected leaves. Thus, all transgenic lines infected with CMV showed cyclic

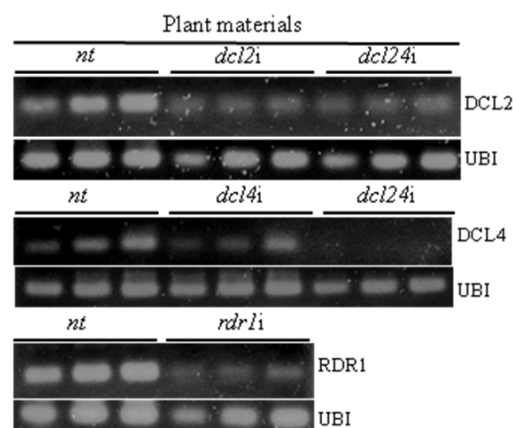


Figure 2. The mRNA expressions of the down-regulated RNA silencing genes, *DCL2*, *DCL4*, *DCL2/4* and *RDR1*, are reduced in the mutant lines. The amounts of DCLs or RDRs mRNA were analyzed by semi-quantitative RT-PCR. A smaller DNA band size was detected in the PCR products in the down-regulated transgenic lines compared with non-transgenic (nt) plants. The PCR products resulting from amplification using a graded number, from left to right, of 28, 30, and 32 cycles are shown. UBI: Ubiquitin gene.

Table 2. Cyclic symptoms on fully expanded leaves of transgenic lines inoculated with CMV.

LeafNo.	Symptom patterns in transgenic lines			
	<i>dcl2i</i>	<i>dcl4i</i>	<i>rdr1i</i>	<i>dcl24i</i>
L5	☐	-	-	☐
L6	☐	☐	☐	☐
L7	☐	☐	☐	☐
L8	☐	☐	☐	☐
L9	■	■	☐	☐
L10	■	■	☐	■
L11	■	■	■	☐
L12	■	■	☐	■
L13	-	■	■	■

L0 is an inoculated leaf. The leaves above L0 were sequentially numbered and the progress of symptoms on fully expanded leaves (8 to 9 cm in length) at 30 dpi observed. ☐: mottle symptom, ■: mosaic symptom, ☐: symptomless leaf.

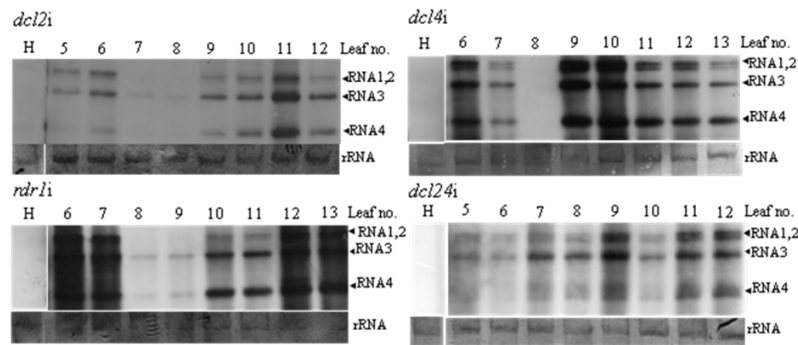


Figure 3. Northern blot analysis of CMV vRNA extracted from YDL tissues from 5-25 dpi in transgenic tobacco lines. Viral RNA was detected with DIG-labeled riboprobe complementary to 3'-UTR sequence. rRNA was stained with methylene blue (MB) as a loading control.

mosaic symptoms on systemic leaves, similar to those in non-transgenic plants (Sunpapao *et al.*, 2011).

### 3.3 Shift of viral RNA amount in CMV-infected YDLs of transgenic lines

Northern blot analysis using a probe specific for CMV viral RNA (vRNA) showed that the amounts of vRNA in the YDLs shifted along the leaf positions all the transgenic lines (Figure 3). There was no obvious difference in the characteristics of the fluctuation of the vRNA amounts in YDLs among the single down-regulated transgenic lines and the non-transgenic plants (Sunpapao *et al.*, 2011). In the *dcl2i* lines, high amounts of CMV vRNA were detected at L5-L6, whereas at L7-L8 no signals were detected, but the vRNA was again clearly present from L9-L12. The fluctuation of the viral concentration by leaf position determined by vRNA did not match the symptoms (Figure 3 and Table 2). This might be due to differences between test plants: the vRNA was assessed in three individual plants, and the time patterns of vRNA in young developing leaves may vary between plants. In the *dcl4i* lines, high levels of CMV vRNA amounts were detected at L6, lesser amounts at L7-L8, high again at L9-L10, then reduced again at L11-L13. In the *rdr1i* lines, high levels of CMV vRNA were detected at L6-L7, lesser at L8-L9, with increase back to high amounts at L10-L13. The above patterns are consistently similar between the three transgenic lines, in terms of having comparatively low vRNA amounts around L8, which corroborates this as a non-random phenomenon. In contrast, no obvious characteristic fluctuation pattern of vRNA amount in YDLs was observed in the *dcl24i* lines.

### 3.4 mRNA expressions of RNA silencing components during CMV infection

The mRNA amounts of *DCL2*, *DCL4*, and *RDR1* in *dcl2i*, *dcl4i*, *dcl24i*, and *rdr1i* lines were not different among uninfected healthy leaves, highly infected leaves or leaves with low infection (Figure 4). The expression of *DCL2* or

*RDR1* in highly infected leaves of *dcl2i* (L11 & L12) or *rdr1i* (L12 & L13) was slightly restored compared to uninfected leaves, respectively. On the other hand, the expression of *DCL2* or *RDR1* in the leaves with low levels of infection of the *dcl2i* (L7 & L8) or *rdr1i* (L8 & L9) lines was not different from uninfected leaves (Figure 4), confirming that the reduction of viral amounts in the YDLs of *dcl2i* or *rdr1i* (Figure 3) was not due to the restoration of *DCL2* or *RDR1* expression.

## 4. Discussion

In this study, we investigated the role of RNA silencing components in the development of cyclic mosaic symptoms from infection by CMV. Based on the visual symptoms, the reduction of RNA silencing components *DCL2*, *DCL4*, and *RDR1* alone was not able to change the cyclic mosaic expression in plants inoculated with CMV (Table 2). Northern blot analysis revealed that the change of vRNA in single down-regulated lines was associated with cyclic mosaic development, but in the double down-regulated line it was not (Figure 3).

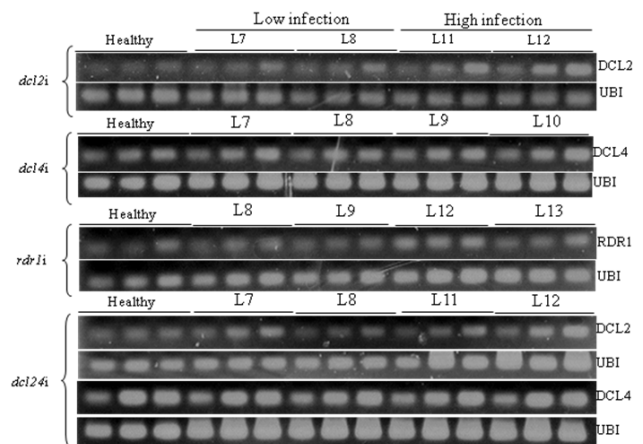


Figure 4. Semi-quantitative RT-PCR analyses of mRNA expressions in transgenic tobacco lines with low and high infections of CMV. (UBI = Ubiquitin gene, used as a control.)

Recently, Fukuzawa *et al.* (2010) reported that the expression of HC-Pro, a potyvirus RNA silencing suppressor in *Nicotiana benthamiana*, canceled cyclic symptom expression upon CMV infection. The authors hypothesized that the host defense conferred by RNA silencing was an essential factor in determining CMV cycling. Therefore, we examined the role of the RNA silencing components *DCL2*, *DCL4*, and *RDR1*, in inducing CMV cyclic symptom expression using down-regulated lines. The single down-regulated lines *dcl2i*, *dcl4i* and *rdr1i*, and the double down-regulated line *dcl24i* expressed cyclic symptoms similar to the non-transgenic tobacco plants (Sunpapao *et al.*, 2011). In this study, we used transgenic lines in which the mRNA expression of the RNA silencing components had been down-regulated by using inverted-repeat constructs. mRNAs expression of RNA silencing components was not completely eliminated (Figures 2 and 4). Therefore, the low expression of these genes might have been enough for cyclic mosaic symptom expression.

In many cases, a single mutation of DCLs results in hardly any change in the RNA silencing deficiency phenotype (Bouché *et al.*, 2006; Deleris *et al.*, 2006; Fusaro *et al.*, 2006). In fact, Diaz-Pendon *et al.* (2007) showed that a single mutation of DCL2 or DCL4 in *Arabidopsis* hardly affected the symptom phenotypes by 2b defective CMV ( $\Delta$ 2b) mutant while higher accumulation levels and more severe disease symptoms induced by  $\Delta$ 2b infection resulted from double mutation of both DCL2 and DCL4. Also, a single mutation of RDR1 hardly affected the symptom phenotypes by  $\Delta$ 2b infection (Wang *et al.*, 2010; 2011). Similar reports have been published on TCV, *Tobacco rattle virus*, and CMV (Bouché *et al.*, 2006; Deleris *et al.*, 2006; Fusaro *et al.*, 2006). From these reports, single down-regulation of an RNA silencing component might not cancel the cyclic mosaic symptom expression caused by CMV infection, because of the hierarchical action of other genes. In the double down-regulated line *dcl24i*, no obvious shift of viral amounts in YDLs was observed, although *dcl24i* exhibited cyclic mosaic symptom expression. Since DCL2 and DCL4 are the main components of antiviral RNA silencing (Deleris *et al.*, 2006) their simultaneous down-regulation might affect the accumulation of viral RNA slightly, but had no effect on the cyclic mosaic symptom phenotype.

The CMV-encoded 2b protein specifically interferes with the RDR6 dependent synthesis of secondary viral siRNAs (Wang *et al.*, 2011). Hence, there is a possibility that the silencing suppressor prevents different steps in the RNA silencing processes, and that the assays using wild-type virus and RNA silencing down-regulated lines in this study did not detect any differences. Recent studies have shown that the use of viral suppressor-deficient mutant viruses facilitates the examination of the genetic requirements of antiviral RNA silencing (Li *et al.*, 2002; Deleris *et al.*, 2006; Diaz-Pendon *et al.*, 2007; Martín-Hernández and Baulcombe, 2008; Wang *et al.*, 2011), although  $\Delta$ 2b mutant of CMV

showed asymptomatic phenotype but not cyclic mosaic symptom.

### Acknowledgements

We thank Dr. T. Meshi of Division of Plant Sciences, National Institute of Agrobiological Sciences, Japan, for kindly providing RNA-depressed tobacco plant materials. The copy-editing service of Research and Development Office (RDO) of Prince of Songkla University (PSU), especially the comments and suggestions of Assoc. Prof. Seppo Karrila, are gratefully acknowledged.

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