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The effect of the decreased expression of dicer-like proteins 2 and 4 on cyclic viral titer, and short interference RNA in *Cucumber mosaic virus* infected tobacco plants

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When infected with *Cucumber mosaic virus* (CMV), infected tobacco plants developed cyclic patterns of mosaic and mottle symptoms interspersed with symptomless leaves along the stem of individual plants. To determine the virus-plant interaction, the CMV viral coat protein (CP) and short interference RNA (siRNA) in CMV-infected tobacco were analyzed. A cyclical accumulation of CP was detected in young developing leaves of non-transgenic plants, but not in DCL2/4i transgenic plants. Northern blot analysis showed that CMV-specific siRNA of young developing leaves varied based on leaf position, and was not related to symptom severity in fully expanded leaves.

Key words: Coat protein, *Cucumber mosaic virus*, mosaic, RNA silencing

Introduction

Cucumber mosaic virus (CMV) is a member of the genus *Cucumovirus* in the family *Bromoviridae*. CMV is widespread around the world and causes serious yield losses in important crops. This virus has a wide range of hosts (Palukaitis and Gracia-Arenal, 2003) and attacks a greater number of vegetable varieties, ornamentals, weeds, and other plants than do other viruses. Most characteristic among the symptoms induced by CMV are foliar mosaic, leaf deformation, and varying degrees of stunting (Palukaitis *et al.,* 1992). Previously, the kinetics of infection by the wild-type CMV (Pepo) during leaf morphogenesis i.e. the infection of leaf primordia (LP) and the developing stages of non-transgenic tobacco plants were examined. The distribution of CMV in fully expanded symptomless leaves and leaves bearing the mosaic

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symptom during the late infection period was determined at the LP stage (Sunpapao *et al.,* 2011). It was found that the 2b protein of CMV was required to establish a CMV infection of meristem tissues and to induce cyclic symptoms on the infected non-transgenic tobacco plants (Sunpapao *et al.,* 2009).

Plants have no immune system to battle viral infections as animals do, but they have a special defensive system. Virus infections in plant hosts activate an antiviral mechanism known as RNA silencing, which is based on sequence-specific degradation of RNA (Ding and Voinnet, 2007). This is an adaptive defense mechanism that is triggered by double stranded RNA (dsRNA) corresponding to the invading viral RNA. Subsequently, the dsRNA is cleaved into small fragments called short interference RNA (siRNA) by RNase III-like enzymes such as dicer and dicer-like protein (DCL) (Voinnet, 2005). It is known that the *Arabidopsis thaliana* genome encodes four DCL enzymes, six RNA dependent RNA polymerases (RDRs) and other components of the silencing machinery in plants and the *Nicotiana tabacum* genome encodes four DCL and six RDR homologues. To counteract this host defense mechanism, many RNA and DNA plant viruses encode suppressor proteins (Roth *et al.,* 2004) including CMV 2b protein (Brigneti *et al.,* 1998).

Although the only available strategy beyond classical plant breeding for the control of virus diseases in crops is the use of virus-tolerant varieties, study of the interaction between RNA silencing and viral distribution in the occurrence of symptoms is valuable. The present study compared viral titer and siRNA amounts in young developing leaves and the pattern of symptoms in CMV-infected non-transgenic and DCL2/4i transgenic plants.

Materials and methods

Plant materials, virus and inoculation

The tobacco plants used in this study were bred from plants provided by Dr. T. Meshi of the Division of Plant Sciences, National Institute of Agrobiological Sciences, Tsukuba. The tobacco plants were down-regulated by using inverted-repeat constructs, which is an RNA silencing based mechanism. The RNA interference (RNAi) knock-down tobacco plants, double mutant DCL2/4i were then used as plant materials. The Pepo strain of CMV was originally obtained from *Cucurbita pepo* (Osaki *et al.,* 1973). The largest leaves of the five-to seven-leaf-stage of transgenic and non-transgenic tobacco plants were mechanically inoculated with purified CMV (25 µg/ml). The inoculated leaf was defined as leaf position 0 (L0), while the leaves above the inoculated leaf were sequentially numbered. The inoculated plants were grown under

greenhouse conditions, and then symptoms in the fully expanded leaves were observed for a month.

Western blot analysis

Total protein was extracted from young developing leaf tissues samples with homogenate buffer (250 mM Tris-HCl pH 7.5, 2.5 mM EDTA, 0.1% ascorbic acid, 1mM phenylmethylsulphonylfluoride). The extracted protein samples were separated in 10% SDS-acrylamide separating gel and stacking gel and were transferred to nitrocellulose membranes. The procedures for detecting the immuno-method and signal were performed as described previously (Sunpapao *et al.,* 2011).

Northern blot analysis

Total RNA was extracted from young developing leaves (1 to 2cm) by using Tripure Isolation Reagent (Roche Diagnostics, Tokyo, Japan) according to the manufacturer's instructions. For siRNA detection, 20µg total RNA was dissolved in an equal volume of 100% formamide and loaded onto a 15% acrylamide gel containing 7 M urea. The low-molecular-mass RNAs were transferred onto a Hybond-NX membrane (GE Healthcare Biosciences) by electroblotting. Hybridization was performed using DIG-labelled, full length CMV RNAs complementary to the CMV negative-sense RNAs transcribed from Pepo CMV clones (Saitoh *et al.,* 1999). The procedures for hybridization and the detection of signals were performed as described previously (Mochizuki *et al.,* 2004).

Results

Cyclic symptom patterns of CMV-infected tobacco

To analyze the pattern of mosaic symptoms, non-transgenic and DCL2/4i tobacco plants were inoculated with CMV and the symptoms were then observed 30 days post-inoculation. The results showed that all the plants developed cyclic mosaic patterns (Table. 1).

Table 1. Pattern of symptoms in non-trnasgenic (Nt) and DCL2/4i transgenic tobacco plants infected with CMV

Plants	Leaf positions (L)										Patterns
	L5	L6	L7	L8	L9	L10	L11	L12	L13	L14	
Nt	-	-	M	M	Mo	S	S	S	M	M	Cyclic
DCL2/4i	Mo	Mo	Mo	S	Mo	M	Mo	M	-	-	Cyclic

M = mosaic, Mo = mottle, S = symptomless

Cyclical accumulation of viral titer

Because the expression of mosaic symptoms has been implicated in the distribution of the virus in young developing leaves, the study focused on the viral coat protein (CP) in such leaves, 1 to 2cm in length, at all leaf positions in the CMV-infected non-transgenic and DCL2/4i tobacco plants. The viral CP in young developing leaves was established by western blot analysis. In the non-transgenic plants, the cyclical accumulation of viral CP varied based on leaf position (Fig. 1). High levels of CP were detected at L7 and L8, reduced levels at L9-L11, restored high levels at L12 and L13 which decreased again at L14. No cyclical accumulation of CMV CP was detected in DCL2/4i. This result suggested that double down regulation of both DCL2 and DCL4 might break the cyclical accumulation of viral titer in DCL2/4i transgenic plants.

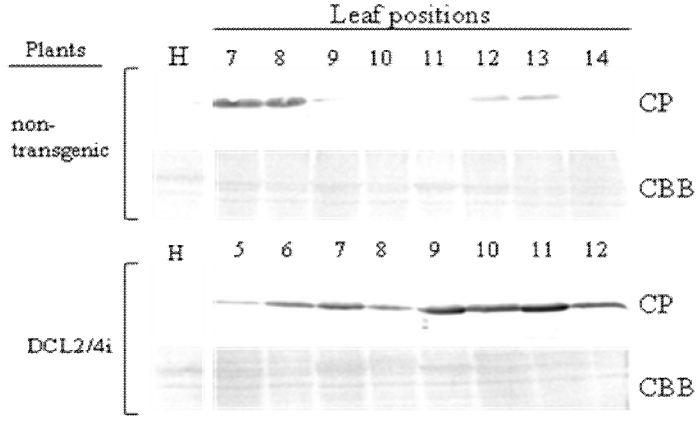

Fig. 1. Accumulation of CMV CP in young developing leaves, 1 to 2cm in length in CMV infected tobacco plants. The numbers indicate leaf positions above the inoculated leaf (L0). The amounts of viral CP were analyzed by western blot analysis. CMV CP was detected. with an anti-CMV IgG antibody. Total proteins were stained with coomassie brilliant blue (CBB) to ensure equal loading. H: healthy plant.

Accumulation of CMV-specific siRNA

CMV-specific siRNA can be used as a general indicator of RNA silencing against viral infection, therefore the amounts of siRNA derived from CMV viral RNA were analyzed. After inoculation with CMV, three young developing leaves were sampled from different plants. The amounts of siRNA in the leaf tissues were examined by northern blot analysis (Fig. 2). In the non-transgenic tobacco plants, siRNA peaked at L7-L9, and L11-L13. In the DCL2/4i transgenic tobacco plants, siRNA was detected from L5-L12 which peaked at L11 and L12. These results suggest that the amounts of siRNA in young developing leaves are not related to the mosaic patterns in the expanded leaves of either non-transgenic or transgenic tobacco plants.

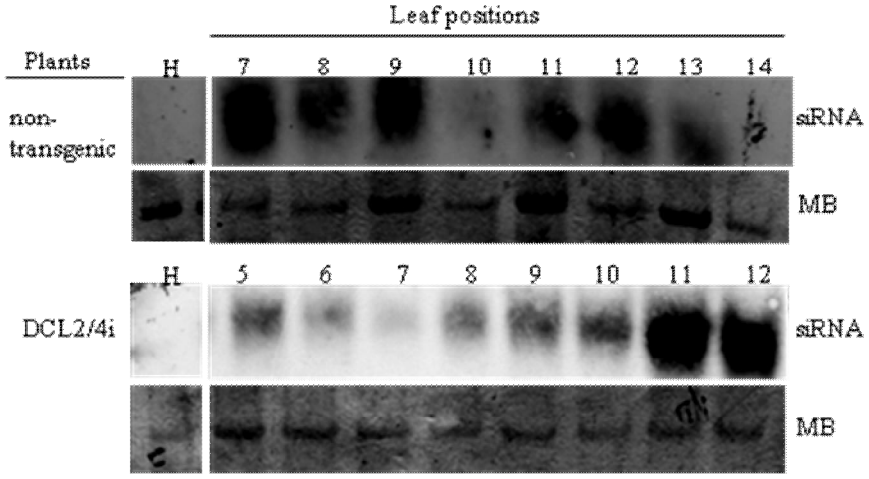

Fig. 2. Northern blot analysis of CMV-specific siRNA corresponding to RNA extracted from young developing leaves of CMV-inoculated tobacco plants, 10, 15, 20 and 25 days post-inoculation. Total RNA was extracted from three young developing leaves and 20μg total RNA was analyzed to detect siRNA. siRNA was detected based on full-length negative-sense CMV RNA. MB: methylene blue staining.

Discussion

Following CMV infection, some fully expanded tobacco leaves display mosaicism, whereas other leaves recover and are symptomless (Ohki *et al.,* 1990), and this is known as cyclic symptom expression. Previous studies conducted by the present researchers showed that viral CP and viral RNA in young developing leaves shifted with leaf position and was related to symptom severity on the fully expanded leaves in non-transgenic tobacco plants

(Sunpapao *et al.,* 2011). In this study the viral titer and the amounts of siRNA were compared between non-transgenic and double down-regulated transgenic (DCL2/4i) plants. The symptom pattern of both the non-transgenic and DCL2/4i transgenic plants was not significantly changed (Table. 1). Although there was not a complete loss-of function, the lower expression of both genes might have been sufficient for cyclic symptom expression.

The double down regulation of both DCL2 and DCL4 broke the cyclical accumulation of viral CP (Fig.1). A previous study (unpublished data) showed that the low expression of DCL2, DCL4 and RDR1 does not affect viral titer, and there was no difference between non-transgenic and transgenic plants. To establish RNA silencing against CMV infection, CMV-specific siRNA in CMV-infected tobacco plants was detected as a general indicator of RNA silencing. Thus viral CP in young developing leaves seemed to be mostly paralleled by the symptom severity in expanded leaves, while siRNA was not. Further, CMV has been to known to encode RNA silencing suppressor protein (2b protein) which interferes with several steps in the antiviral RNA silencing pathway resulting in varying amounts of siRNA in young developing leaves. CMV 2b protein might disrupt the amount of siRNA in the RNA silencing mechanism. However, if there is no completed down regulation of DCL2 and DCL4, the silencing components of other genes such as DCL1 and DCL3 may work hierarchically causing cyclic mosaic patterns in the CMV-infected plant host.

In this study it was shown that a reduction of both DCL2 and DCL4 mRNA influenced the cyclical accumulation of CMV titer in DCL2/4i transgenic tobacco plants, but had no impact on the amount of siRNA and the cyclic mosaic patterns.

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