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Movement of *Zucchini yellow mosaic virus* Involved in Symptom Severity on Zucchini Squash

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Zucchini squash (*Cucurbita pepo* cv. Black Beauty) plants infected with A strain of *Zucchini yellow mosaic virus* (ZYMV-A) isolated from a hollyhock plant showed systemically severe mosaic symptom, similar to previously established Cu strain of ZYMV. However, initial symptom of squash infected by ZYMV-A strain was generally more severe than those infected by ZYMV-Cu. Using leaf-detachment assay, examination of kinetics of accumulation of the coat protein (CP) in systemic leaves of squash plants showed that CPs of ZYMV-A appeared earlier than those of ZYMV-Cu. However, both ZYMV-A and ZYMV-Cu showed similar kinetics of CP accumulation 7 days post-inoculation. These results indicate that different rates and initial severity of systemic symptom development were due to differences in the rate of movement rather than virus replication.

Keywords : Movement, *Potyvirus*, severity, squash, symptom, *Zucchini yellow mosaic virus*.

Plants have evolved to fight viral infection through expression of resistance genes that are effective against invading viruses or hypersensitive cell-death to block virus spread (Baker et al., 1997). In turn, viruses use a variety of strategies to promote their infections in susceptible hosts. These virus strategies are involved in physiological modifications to host cells that enhance infection such as the formation of replication complexes (Hills et al., 1987), suppression of post-transcriptional gene silencing (Baulcombe, 2002), interference with regulation of the plant cell cycle (Gutierrez, 2000), and alteration of cell-to-cell trafficking or long-distance movement (reviewed by Lazarowitz and Beachy, 1999; Lucas and Wolf, 1999). These effective movement functions of potyviruses are related to symptom severity and determination of host specificity (Revers et al., 1999).

Zucchini yellow mosaic virus (ZYMV), a member of the genus *Potyvirus* in the family *Potyviridae*, was first reported in Italy and France by Lisa et al. (1981). ZYMV has a filamentous virion particle of about 800 × 12 nm and a monopartite genome containing a messenger-sense single-stranded RNA, encoded a single polyprotein processed subsequently into 10 proteins at specific point by virus-encoded proteases (Lisa et al., 1981). The virus became a major pathogen in cucurbit plants throughout the world (Provvidenti et al., 1984).

ZYMV A strain (ZYMV-A) isolated originally from hollyhock plant (Choi et al., 2002b) induced highly severe yellow-green mosaic and leaf malformation symptoms on cucurbit plants 7-10 days post-inoculation (dpi) and facilitated a movement-deficient M strain of *Cucumber mosaic virus* to systemic movement in zucchini plant (Choi et al., 2002a). Although symptoms of zucchini squash infected by Cu strain of ZYMV (ZYMV-Cu) were similar to systemic symptoms of ZYMV-A (Yoon and Choi, 1998), initial symptom severity of ZYMV-Cu was relatively milder than that of ZYMV-A (Choi et al., 2002b).

This study showed that movement kinetics of ZYMV is involved in early symptom expression in zucchini squash.

Materials and Methods

Plant growth and virus inoculation. ZYMV strains, ZYMV-A and ZYMV-Cu, used in this study were propagated in leaves of cucumber (*Cucumis sativus* cv. Baekdadaki) plants by mechanical inoculation with 10 mM sodium phosphate buffer (pH 7.0). Cotyledons of squash plants (*Cucurbita pepo* cv. Black Beauty) were mechanically inoculated with sap extracts from the ZYMV-A and ZYMV-Cu infected leaves of cucumber plants. The inoculated plants were grown in the greenhouse with controlled temperature of 26°C.

Leaf detachment assay and western blot hybridization. ZYMV-inoculated cotyledons were excised to assess the kinetics of systemic movement from 3 to 10 dpi. Total proteins were extracted with 1X Tris-EDTA buffer containing 2% SDS from systemic leaves infected by ZYMV, and then centrifuged at 10000 × g. The supernatant was extracted with three-time phenol: chloroform (24:1) and subjected to 12% SDS-polyacrylamide gel electrophoresis (PAGE). The blotting steps on nitrocellulose

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membrane were performed according to the manufacturers instruction (Bio-Rad, USA). After electroblotting, blotted proteins were immunodetected with the antiserum against ZYMV CP (diluted in 1:1000). Subsequently, the membranes were incubated with the solution of goat anti-rabbit IgG (diluted in 1:7,500, Promega, USA) antibody conjugated with alkaline phosphatase as the secondary antibody, and color reaction was developed with Western Blue Stabilizer Substrate (Promega, USA).

Results and Discussion

A hollyhock (*Altheae rosea*)-infecting virus was identified as a member of the genus *Potyvirus*, which was named as ZYMV-A on the basis of electron microscopy and RT-PCR analysis (Choi et al., 2002b). With respect to symptomatology of ZYMV-A, squash plants inoculated with ZYMV-A showed systemically severe blistering mosaic and yellowing, highly similar to plants infected with ZYMV-Cu. However, ZYMV-A was able to produce more quickly systemic symptom on cucumber and zucchini squash (*Cucurbita pepo* cv. Black Beauty) than ZYMV-Cu. This, on the other hand, does not mean any difference in the kinetics of virus replication or movement, but only in the timing of symptom appearance. Thus, the squash plants inoculated with either ZYMV-A or ZYMV-Cu were analyzed at different dpi for the accumulation of coat protein (CP). The CP accumulation pattern on the systemic leaves of squash plants was analyzed by using leaf detachment assay. The inoculated cotyledons were excised to block systemic spread in squash plants at different days post-inoculation. The total proteins were extracted from upper leaves and were subjected to western blot assay using CMV-specific antiserum.

In systemic leaves, the CP of ZYMV-A was first detected at 3 dpi, at which time the first true leaves had emerged (Fig. 1A). The amount of CPs for A strain reached a plateau at 6 dpi and was maintained at relatively constant concentrations of up to 10 dpi (Fig. 1A). Meanwhile, the CP accumulation of ZYMV-Cu was first detectable at 5 dpi in systemic leaves (Fig. 1B). The CP levels of Cu strain at 7 dpi were comparatively similar to 10 dpi in squash plants (Fig. 1B), indicating that the CP accumulation of ZYMV-Cu reached a plateau at 7 dpi. In addition, CP accumulations of both ZYMV strains greatly decreased after 14 dpi (data not shown), and it appeared that host plants did not grow poorly or die. Thus, the kinetics of accumulation of the two strains of ZYMV was different in systemically infected leaves.

Since analysis of the kinetics of accumulation of the CP in squash plants infected by ZYMV-A vs. ZYMV-Cu showed no difference at 7 dpi, it can be said that systemic symptoms of squash plants inoculated with ZYMV-A were

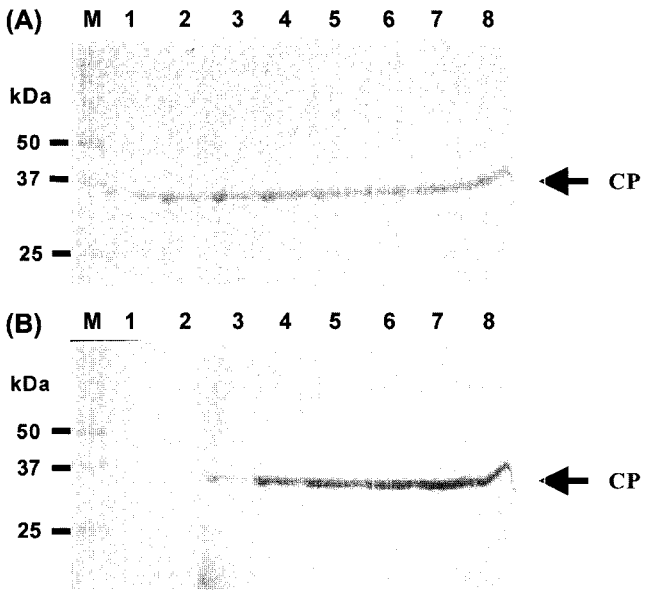

Fig. 1. Capsid protein accumulation in zucchini squash plants infected by ZYMV-A (**A**) and ZYMV-Cu strain (**B**), as a time course. The cotyledons of zucchini squash were inoculated with ZYMV-A and ZYMV-Cu, then the cotyledons were cut to assess for rate of long-distance movement of the viruses from 3 day post-inoculation (dpi) to 10 dpi. M: protein size marker (Bio-Rad); 1-8: cut from 3 dpi to 10 dpi. The total protein was extracted from the upper leaves at 14 dpi and separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and were immnunodetected by western blot assay using antiserum specific to ZYMV capsid protein. The positions of capsid protein (CP) are indicated, and relative molecular weight of size marker in kilodaltons is marked adjacent to the panel.

highly similar to those of plants infected by ZYMV-Cu after 7 dpi. This suggests that symptom severity is correlated with CP accumulations of ZYMV in zucchini squash plant. Similarly, CP accumulation pattern for ZYMV-A in leaf-detached plants showed highly similar kinetics of accumulation in undetached zucchini squash (data not shown). These data again show a difference in the rate of systemic infection by ZYMV-A vs. ZYMV-Cu, with a lag of 2 days for ZYMV-Cu between the systemic movement of the infectious virus and viral-specific products in systemic leaves. Although the difference in the rate of cell-to-cell movement between two ZYMV strains cannot be excluded, it is likely that systemic movement of ZYMV-A is crucial for initial symptom severity, on the basis of detection time of their CPs in systemic leaves of squash plants. These results suggest that coding proteins or RNA itself of ZYMV-A strain helps in the fast formation of RNA-protein complex of the virus to enter the vasculature system of the host plants for systemic movement, rather than viral replication.

In general, potyvirus-encoded gene products associated with virus accumulation have been demonstrated in many

potyviruses that infect tobacco, particularly focused on HC-Pro and CP. Peng et al. (1998) demonstrated that the amino acid substitutions within FRKN motif of HC-Pro gene of ZYMV-WK strain initially expressed mild symptom, which changed from mild to severe symptom along with increasing virus accumulation. In tobacco, two mutants in C-terminal region of HC-Pro gene of *Tobacco vein mottling virus* (TVMV) also caused a delay of symptom expression and a lower virus accumulation (Cronin et al., 1995). However, ZYMV-AG strain, an attenuated ZYMV-NAT strain that has been replaced by Ile for Arg at position 180 in conserved FRKN motif, was shown to affect the severity of symptom, but did not cause virus accumulation in cucurbits (Gal-On, 2000). While this study did not determine the nucleotide sequences of HC-Pro gene for two ZYMV strains, HC-Pro of ZYMV-A might affect the rate of viral movement together with other viral coding proteins, resulting in the rapid CP accumulation of ZYMV-A which induced faster systemic symptom than that of ZYMV-Cu due to rapid systemic movement. Alternatively, HC-Pro of ZYMV plays an important role as suppressor to post-transcriptional gene silencing to defense factors of host plant, as demonstrated by HC-Pro of *Tobacco etch virus* (Kasschau and Carrington, 1998).

This study demonstrated that kinetics of accumulation of ZYMV-A was correlated with initial symptom severity. The accumulation of all ZYMV-A-encoded proteins reached a plateau at 3 dpi, while those encoded by ZYMV-Cu continued to increase. Subsequently, it is likely that accumulation more than threshold required for symptom expression is easily reached and severe systemic symptoms in squash plants are produced. This indicates that a threshold level of virus accumulation is required to induce systemic symptom. With CP relation to symptom expression and virus accumulation, Adrejeva et al. (1999) replaced amino acids within CP in *Potato virus* A (PVA) to reduce the virus accumulation on tobacco, and demonstrated the simultaneous mutants in HC-Pro and amino acid replacement in CP to delay the systemic movement, changing into a mild symptom. This implied the coordinated functions of HC-Pro and CP in accumulation and movement as shown by TVMV, PVA, and other potyviruses (Rojas et al., 1997). There have been many reports that a threshold level of virus accumulation is required to induce the systemic symptom in various plants. As an example, rapid CP and RNA accumulations of a severe strain of *Cucumber mosaic virus* (CMV; Fny-CMV) caused severe symptom via both fast cell-to-cell movement and long-distance movement on zucchini squash, compared to that of Sny-CMV (Gal-on et al., 1994). It is of interest to note that systemic symptoms of squash plants induced by ZYMV-A isolated from a weed plant, hollyhock, were more severe than those of plants infected with ZYMV-Cu originally isolated from cucumber, a ZYMV-common host (Yoon and Choi, 1998). Further research work is needed to determine the factors affecting the movement of ZYMV in cucurbits.

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