Cucumber mosaic virus* Establishes Systemic Infection at Increased Temperature Following Viral Entrance Into the Phloem Pathway of *Tetragonia expansa

Takashi Kobori, Takeshi Osaki, and Satoshi T. Ohki

Graduate School of Agriculture and Biological Sciences, Osaka Prefecture University, Sakai, Osaka 599-8531, Japan.

Current address for T. Kobori: Kyoto Prefectural Institute of Agricultural Biotechnology, Seika-cho, Kyoto 619-0244, Japan.

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ABSTRACT

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A potential regulatory site for *Cucumber mosaic virus* (CMV, pepo strain) movement necessary to establish systemic infection was identified through immunological and hybridization studies on *Tetragonia expansa*, which was systemically infected by CMV at 36°C but not at 24°C. In inoculated leaves, cell-to-cell movement of CMV was enhanced at 36°C compared with that observed at 24°C. CMV was distributed in the phloem cells of minor veins as well as epidermal and mesophyll cells at

both 36 and 24°C. CMV was detected in the petioles of inoculated leaves, stems, and petioles of uninoculated upper leaves at 36°C, whereas CMV was detected only in the petioles of inoculated leaves and in stems at 24°C. CMV moved into the phloem and was transported to the stem within 24 h postinoculation (hpi) at 36°C. However, it did not accumulate in the petioles of the upper leaves until 36 hpi. In petioles of inoculated leaves at 24°C, CMV was detected in the external phloem but not in the internal phloem. From these results, we conclude that systemic infection is established after viral entrance into the phloem pathway in *T. expansa* at 36°C.

Additional keywords: long-distance virus movement, resistance mechanism.

Systemic infection of plant viruses depends on several processes. Viruses replicate in an initially infected cell and then move to adjacent cells through plasmodesmata (cell-to-cell movement). Moving through mesophyll cells, the viruses enter sieve elements through bundle sheath cells, phloem parenchyma cells, and/or companion cells and then are transported to other organs and tissues through the phloem (long-distance movement). These processes have, to date, been extensively reviewed (4,13,21,22,26).

Cell-to-cell or long-distance movement of viruses is a key factor in determining viral host range, and the inhibition of either movement is a common resistance mechanism to viral infection. Restriction of long-distance movement may be caused by the inhibition of loading into the phloem, transportation through the phloem, or unloading from the phloem to nonvascular tissues. The restriction of viral movement into phloem was shown at bundle sheath cell-phloem cell interfaces in some cases: *Cucumber mosaic virus* (CMV) in *Cucumis figareii* (20), *Cowpea chlorotic mottle virus* in a resistant soybean line (14), *Tomato aspermy virus* in cucumber (32), and CMV in a transgenic tobacco exhibiting replicase-mediated resistance (38). The restriction after phloem entry was reported for *Tobacco etch virus* in a resistant tobacco line (25), *Pepper mottle virus* (PepMoV) in a resistant pepper (15), CMV in a resistant pepper (11), *Carnation mottle virus* in *Chenopodium quinoa* under growth chamber conditions (12), *Tobacco mosaic virus* (TMV) lacking its coat protein (CP) (10), and *Turnip vein clearing virus* in tobacco treated with cadmium at a nontoxic concentration (6).

CMV is a 30-nm icosahedral plant virus consisting of single-stranded, positive-sense RNA and has three genomic RNAs. RNA

3 has two open reading frames that encode 3a protein and CP. The 3a protein is essential for cell-to-cell movement and is referred to as the movement protein (7,18,37). CP is also required for cell-to-cell movement (2,3,28). Both 3a protein and CP are necessary for long-distance movement (18,31). The 2b protein, encoded by RNA 2, also is reported to affect systemic movement (9,27).

Tetragonia expansa, family Aizoaceae, shows only local infection at 24°C, but is systemically infected at 36°C, with the pepo strain of CMV. The systemic movement under high temperature was determined by one amino acid at position 129 in CP, which is encoded by CMV RNA 3 (19). However, regulatory sites for CMV movement necessary to establish systemic infection have not been determined. In this paper, we report that the systemic accumulation of pepo-CMV is restricted after entry into the phloem at 24°C in *T. expansa*; possibly caused by the inhibition of viral transport from the external phloem to the internal phloem.

MATERIALS AND METHODS

Plants, CMV strains, and CMV inoculation. *T. expansa* was grown in growth chambers (NK Systems, Osaka, Japan) with a 16-h light/8-h dark cycle. *T. expansa* plants grown to the five- to seven-leaf-stage were used for inoculation and incubated at 24 or 36°C. Pepo-CMV was used in this study (23). CMV was propagated in tobacco (*Nicotiana tabacum* cv. Xanthi) and purified as described previously (30). The one or two largest leaves of the *T. expansa* plants were mechanically inoculated with purified virus at a concentration of 100 µg/ml.

Tissue fixation, embedding, and sectioning. Tissue fixation procedures were performed as described by van Lent and Verduin (36) with minor modifications. Leaf samples (1 × 10 mm) that contained chlorotic tissue from inoculated leaves and petioles (5 mm long) were obtained at 5 days postinoculation (dpi). The tissue samples were fixed under vacuum in 1% (wt/vol) glutaralde-

Corresponding author: S. T. Ohki; E-mail address: ohki@plant.osakafu-u.ac.jp

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hyde and 4% (wt/vol) paraformaldehyde in 0.1 M phosphate buffer, pH 7.0, for 30 min to 1 h on ice. After infiltration, fixation was allowed to proceed for 12 h at 4°C. The samples were washed six times for 10 min in buffer and dehydrated in a graded series of ethanol (30, 50, 70, and 90% for 20 min each under vacuum; and 100% twice for 20 min) at room temperature. The ethanol was replaced with LR White Resin (London Resin, Reading, UK) by incubating in a 1:1 mixture of ethanol/LR White for 1 h at room temperature and then pure LR White for 1 h at room temperature followed by an overnight incubation at 4°C in fresh LR White. The tissue samples were transferred to BEEM Conical Tip capsules (Better Equipment for Electron Microscopy Inc., New York), and the resin was polymerized for 16 to 24 h at 55°C. Semithin sections were cut with glass knives on a Leica UCT ultramicrotome (Leica Microsystems AG, Vienna, Austria). The sections were transferred with tweezers onto drops of distilled water and dried onto aminopropylsilane-coated glass slides (Matsunami, Osaka, Japan).

Immuno-histochemistry. Procedures for immuno-histochemistry were as described previously (36). Specifically, the sections were incubated for 30 min at room temperature in 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS: 8.1 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 , 0.137 M NaCl, and 2.7 mM KCl), pH 7.4, in a vertical staining jar. Primary antibody solution (1:200 dilution of mouse monoclonal antiserum prepared against pepo-CMV CP in PBS containing 0.05% Tween 20 and 1% BSA [PBST-BSA]) was dropped onto the sections, and the sections were incubated horizontally overnight at 4°C. After washing the slides three times (10 min each) in PBST, secondary antibody solution (1:40 dilution of immunogold conjugated LM goat anti-mouse [British Biocell, Cardiff, UK] in PBST-BSA) was dropped onto sections, and the sections were incubated for 90 min at room temperature. The slides were washed three times as described previously, fixed for 15 min in 1% (wt/vol) glutaraldehyde in PBS, and washed twice (10 min each) in distilled water. Silver enhancement of gold labeling was conducted by addition of silver

Fig. 1. Leaf press-blot analysis of *Cucumber mosaic virus* (CMV)-inoculated leaves from *Tetragonia expansa* incubated at 24°C (24) or 36°C (36) at 1, 2, and 5 days postinoculation (dpi) (indicated by 1, 2, and 5, respectively). The right half of the inoculated leaves (Leaf) was pressed onto nylon membranes. Viral RNA was probed with a digoxigenin-labeled RNA complementary to the 3' common sequence of CMV positive-sense RNAs. No signal was detected in the mock-inoculated leaf (Mock). These two series (24 and 36) of panels are from the same membrane. Number and size (millimeter with standard error) of the infection foci at 1 and 2 dpi are also indicated.

enhancing solution (IntenSEM; Amersham Biosciences, Uppsala, Sweden) to each section followed by incubation for 8 to 15 min at room temperature. The sections were viewed and photographed with a microscope (Olympus BX-20; Olympus, Tokyo).

Leaf press-blotting and dot blotting. Leaf press-blotting was performed as described by Uchiba et al. (34) with minor modification. The lower sides of the leaves sampled at 1, 2, and 5 dpi were mildly abraded with celite using a toothbrush. After treating in distilled water, the leaves were incubated in W5 solution (29) containing 0.7% cellulase and 0.25% macerozyme (Yakult Pharmaceutical Inc., Tokyo) for 2 h at room temperature. The leaves were transferred onto nylon membrane (Hybond N⁺; Amersham Bio-

sciences) with the treated side down and blotted for 30 min with a vacuum gel dryer (Rapidry-mini; ATTO, Tokyo) at room temperature. The membrane was rinsed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate, dried at room temperature, and baked at 80°C for 2 h.

For dot blotting, total RNA was extracted from the leaves as described previously (23). The total RNA extracts (5 µl) were applied onto a nylon membrane and the membrane was baked at 80°C for 2 h. Digoxigenin-labeled T7 RNA polymerase-derived transcripts from pCP3TS503 (24), complementary to the 3' common sequences of CMV RNA, were used as a probe to detect plus-sense CMV RNAs in leaf press-blotting and dot blotting.

Fig. 2. Immuno-histochemical analysis of **A and B**, transverse and **C and D**, longitudinal sections of inoculated leaves containing fourth- or fifth-order minor veins of *Tetragnia expansa* grown at 24 and 36°C. Samples were excised at 5 days postinoculation and immuno-stained with an antibody against *Cucumber mosaic virus* (CMV) coat protein. Bundle sheath cells (BS) and phloem cells (PC) including phloem parenchyma cells, companion cells, and sieve elements at **A and C**, 36°C and **B and D**, 24°C are indicated. **E**, A vein from healthy control. Arrowheads show phloem cells containing CMV. T = xylem tracheary elements. Bars = 20 µm.

Hybridization and detection were performed as described previously (19).

Reverse transcription-polymerase chain reaction. Total RNA (500 ng) extracts from *T. expansa* tissues were reverse-transcribed to single-stranded cDNA using the primer P3R123Bam (5'-CCGGATCCTGGTCTCTTTTGGAGGCC-3') complementary to the 3' common ends of CMV. About 500 bp of a pepo-CMV CP gene fragment was amplified using the primers pepoCPF (5'-CCTCTAGACTTCTCCGCGAGATTGCG-3': positions 1,166 to 1,183) and P3CPR3/4 (5'-CCGAGCTCTCAAGCTTGAAGTC-CAGATGCA-3': positions 1,724 to 1,742). In the polymerase chain reaction (PCR), 20 cycles of amplification were performed under the following conditions: denaturation at 92°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. The products were electrophoresed through 1% agarose gel and stained with ethidium bromide. The stained gels were viewed and photographed with an ATTO Densitograph AE-6920M (ATTO, Tokyo).

RESULTS

Cell-to-cell movement in the inoculated leaves at 36 or 24°C.

Our previous experiments showed that, in *T. expansa* grown at 36°C, pepo-CMV induced localized yellow spots on inoculated leaves by 2 to 3 dpi and systemic chlorotic spots and ringspots by 5 dpi, but only localized chlorotic spots on inoculated leaves of plants grown at 24°C by 3 to 5 dpi (19). To compare local spread of CMV in the inoculated leaves at 24°C with that at 36°C, we probed leaf press-blot obtained at 1, 2, and 5 dpi, respectively. CMV RNA signal was detected from tissue grown at 36°C as spots at 1 dpi, elongated regions associated with veins at 2 dpi, and massive coverage distributed throughout the leaf at 5 dpi (Fig. 1, 36-1, 36-2, and 36-5). In contrast, CMV RNA only was detected localized within infection loci by 5 dpi at 24°C (Fig. 1, 24-1, 24-2, and 24-5). Less RNA was detected at 24°C than at 36°C (Fig. 1). These results demonstrated that the cell-to-cell movement was facilitated at 36°C versus 24°C.

CMV entry into phloem at 24 and 36°C. Phloem entry in the inoculated leaves is a likely requirement for the establishment of systemic infection. Using immuno-histochemical methods, we examined whether CMV entered the phloem cells at 24°C by observing tissues from within lesions on the inoculated leaves at 24 and 36°C. CMV antigenic signal was detected in epidermal cells, palisade and spongy mesophyll cells, and bundle sheath cells in fourth- or fifth-order veins (minor veins [8]) at both 24 and 36°C. Vascular parenchyma cells and companion cells around sieve elements were indistinguishable by our method of observation (Fig. 2E; 22), and thus, we defined vascular parenchyma cells, companion cells, and sieve elements as phloem cells in this study. CMV was detected in the phloem cells of all the minor veins in the inoculated leaves from plants grown at 36°C (Fig. 2A and C; Table 1). The virus was also detected in the phloem cells of

most of the minor veins from plants grown at 24°C (Fig. 2B and D; Table 1). These observations indicated that CMV entered the phloem tissues of the inoculated leaves from plants grown at 24°C as well as those grown at 36°C, although CMV did not accumulate in systemic tissue from plants grown at 24°C. Whether CMV invaded sieve elements or not remains unclear by the observations.

We then examined CMV accumulation in the petioles of the inoculated leaves, stems, and the petioles of the upper leaves from plants grown at 24°C by tissue printing and northern blotting. The upper leaves analyzed were the second leaf above the inoculated leaf. The stems were the internodes between the inoculated leaf and the upper leaf. No viral RNA signal was detected in these organs (data not shown), and therefore, we used reverse transcription (RT)-PCR for the tissues harvested at 5 dpi. CMV CP gene fragments were detected from the samples taken from the petioles of the inoculated leaves, stems, and the petioles of the upper leaves of plants grown at 36°C (Fig. 3B, lane 36). In contrast, RT-PCR revealed that CMV was present in the petioles of the inoculated leaves and the stems, but not in detectable amounts in the petioles of the upper leaves of plants grown at 24°C (Fig. 3B, lane 24). These findings show that CMV reached the stems but did not accumulate in the petioles of the upper leaves of plants grown at 24°C.

Is Incubation at 36°C necessary for CMV to establish systemic infection? We determined the incubation period for CMV to be transported from the inoculated tissues to the stem in plants grown at 36°C. Inoculated leaves and petioles of plants incubated at 36°C were detached from their stems at 0, 6, 12, 24, 36, 48, or 72 h postinoculation (hpi); CMV RNA accumulation in the second upper leaves was analyzed by dot blotting at 8 dpi. No signal was detected in the upper leaves of the plants in which the inoculated leaves were detached at 0 to 12 hpi. In contrast, signal was detected in the plants in which leaves were detached at and after 24 hpi (Fig. 4A). These results indicated that CMV moved into the phloem of the inoculated leaves and was transported to the stem by 24 hpi at 36°C.

We then ascertained the required incubation period at 36°C for CMV to establish systemic infection in *T. expansa*. CMV-inoculated plants incubated at 36°C were moved to another growth chamber at 24°C at 0, 6, 12, 24, 36, 48, or 72 hpi and were incubated until 8 dpi; the upper leaves were then assayed for CMV RNA by dot blot. CMV was not detected in the upper leaves of the plants moved to 24°C by 24 hpi. On the other hand, CMV was detected in the upper leaves of plants moved to 24°C at and after 36 hpi (Fig. 4B). These results indicated that CMV required 36 h at 36°C to establish a systemic infection even in plants placed back at 24°C.

Fig. 3. Detection of *Cucurbit mosaic virus* (CMV) RNA by reverse transcription-polymerase chain reaction (RT-PCR) in **A**, the petioles of inoculated leaves (IP), stem (S, internode between the inoculated and upper leaves), and the petioles of the second systemic leaves (UP) of *Tetragonia expansa* inoculated with CMV and grown at 24°C or 36°C for 5 days postinoculation. **B**, Total RNA (500 ng) extracted from IP, S, or UP was reverse-transcribed using primers that would amplify the CMV coat protein gene fragment, which is approximately 500 bp long. RT-PCR products were electrophoresed through a 1% agarose gel and stained with ethidium bromide. The numbers 24 and 36 represent CMV-infected *T. expansa* grown at 24 and 36°C, respectively. M: Mock-inoculated *T. expansa* grown at 24°C.

TABLE 1. *Cucurbit mosaic virus* (CMV) accumulation in different cell types in typical vascular bundles of *Tetragonia expansa* leaves from plants inoculated with CMV and grown at 24 or 36°C^a

Temperature	Number of veins analyzed (sampled tissue)	Number of vascular bundles (%)	
		BS cells ^b	Phloem cells ^c
24°C	10 (8)	10 (100)	7 (70)
36°C	14 (10)	14 (100)	14 (100)

^a Data were based on immuno-histochemical detection of CMV coat protein around and within fourth- and fifth-order veins in semithin cross sections of the inoculated leaves. At 5 days postinoculation, tissues containing chlorotic spots were randomly sampled.

^b Veins containing infected bundle sheath (BS) cells.

^c Veins containing infected phloem cells, i.e., phloem parenchyma or companion cells.

Experiments combining both detachment and temperature shift were carried out also. CMV-inoculated *T. expansa* plants in which inoculated leaves were detached and the plants moved from the chamber at 36°C to another chamber at 24°C at 24 hpi developed no systemic infection at 8 dpi (0/15). However, more than half of the plants (12/20) in which inoculated leaves were detached at 24 hpi and the plants moved to the 24°C chamber at 36 hpi exhibited systemic infection. All the plants (25/25) in which inoculated leaves were detached at 24 hpi and the plants kept in the chamber at 36°C showed systemic symptoms. These results indicated that some period of time after entering the phloem cells was required for CMV to establish systemic infection in *T. expansa* at 36°C.

To support these findings, we traced, by RT-PCR, the CMV accumulation in the petioles of the inoculated leaves, the stems, and the petioles of the second upper leaves from plants grown at 36°C at 24, 36, 48, and 72 hpi. CMV was detected in the petioles of the inoculated leaves and the stems but not in the petioles of the upper leaves at 24 hpi (Fig. 5, lane 24). However, the virus was identified in the petioles of the upper leaves at and after 36 hpi (Fig. 5, lanes 36 to 72). This demonstrates that CMV moved from the stems into the petioles of the upper leaves between 24 and 36 hpi at 36°C.

Distribution of CMV in the petiole of inoculated leaves of plants grown at 24 or 36°C. We further observed CMV distribution in the petioles of the inoculated leaves at 5 dpi of plants grown at 24 or 36°C using immuno-histochemistry. Ten petioles each from plants grown at 24 or 36°C were analyzed. Vascular tissue from plants grown at 36°C was slightly larger than that at 24°C. Signal was detected in the external phloem, the xylem parenchyma, and the internal phloem cells from plants grown at 36°C (Fig. 6A). In contrast, CMV was confined to a few cells of the external phloem from plants grown at 24°C. CMV was never

detected in xylem parenchyma cells and internal phloem cells from plants grown at 24°C (Fig. 6B). This strongly suggests that CMV did not move from the external phloem to the internal phloem at 24°C.

DISCUSSION

Systemic resistance of *T. expansa* against CMV infection is temperature-sensitive. The host plant is systemically infected with

Fig. 5. Detection of *Cucumber mosaic virus* (CMV) in the petioles from inoculated leaves (IP), stem (S, internode between the inoculated and upper leaves), and the petioles from systemic leaves above the inoculated leaf (UP) of *Tetragnia expansa*. Tissue samples were taken at 24, 36, 48, and 72 h postinoculation (indicated by 24, 36, 48, and 72, respectively). The plants were incubated at 36°C. Total RNA (500 ng) extracted from IP, S, or UP was reverse-transcribed using primers that would amplify the CMV coat protein gene fragment, which is approximately 500 bp long. Reverse transcription-polymerase chain reaction products were electrophoresed through a 1% agarose gel and stained with ethidium bromide. Mock-inoculation (M) and purified CMV RNA (+) were used as the controls.

Fig. 4. Dot blot analysis of tissue from the second systemic leaves (+2) of *Tetragnia expansa* grown at 36°C from which **A**, *Cucumber mosaic virus* (CMV)-inoculated leaves were detached at different times or **B**, CMV-inoculated *T. expansa* was moved from a 36°C growth condition to a 24°C growth condition for varying times. Total RNA extracted from the upper leaves at 8 days postinoculation was spotted onto nylon membrane and hybridized with the same RNA probe used in Figure 1. These panels show samples spotted on the same membrane. **A**, The inoculated leaves with petioles were cut from the stem at 0, 6, 12, 24, 36, 48, and 72 h postinoculation (hpi). Arrowheads show the time that inoculated leaves were detached. **B**, Plants incubated at 36°C were moved to the chamber at 24°C at 0, 6, 12, 24, 36, 48, and 72 hpi. Arrowheads indicate the time that plants were moved from the 36°C chamber to the 24°C chamber.

CMV at growth treatment of 36°C or greater, whereas it is locally infected when grown at 24°C (19). In this study, we demonstrated that CMV entered the phloem cells of the inoculated leaf but did not accumulate in the petioles of the upper leaves at 24°C and that CMV requires a period at high temperature after entering the stem in order to establish a systemic infection.

Leaf press-blotting demonstrated that both the quantity and size of the infection sites in the inoculated leaves increased more rapidly at 36°C than at 24°C (Fig. 1). This result suggests that the high temperature conditions facilitated the cell-to-cell movement of CMV in the inoculated leaves of *T. expansa*. The efficiency of cell-to-cell movement often is related to that of vascular movement (26). However, the movement through plasmodesmata between mesophyll cells that was facilitated in plants grown at 36°C was not the cause of the systemic infection exhibited by only these plants because CMV also was detected in the phloem cells in the inoculated leaves at 24°C (Figs. 2 and 6; Table 1). The facilitation of cell-to-cell movement at 36°C in this study may, however, influence the establishment of systemic infection, because more virus particles may enter the phloem pathway in plants grown at 36°C. It also might result in the lower percentage of infected cells observed among the phloem cells in the inoculated leaves from plants grown at 24°C compared with those grown at 36°C (Table 1).

Several groups reported the inhibition of systemic movement after entering in phloem (6,11,12,15,25). In *T. expansa*, CMV was transported into the phloem in plants grown at either 24 or 36°C (Table 1; Fig. 2), indicating that systemic movement of CMV was inhibited after entering the phloem tissue in plants grown at 24°C. CMV was transported into the petioles of the inoculated leaves

and the stem but not into the petioles of the upper leaves in plants grown at 24°C (Fig. 3). It is of interest that CMV is transported from the petioles of the inoculated leaves to the stem but not from the stem to the petioles of the upper leaves in plants grown at 24°C.

The transportation pathway of viruses from petioles to stems is presumably different from that of stems to petioles. The phloem in the major veins of leaves, petioles, and stems is divided into external and internal phloem in some plant families including Aizoaceae. This structural differentiation has functional consequences for the photoassimilate transport (33). The external phloem is used for phloem loading and the internal phloem is used for phloem unloading to uninfected leaves in *PepMoV-C. annuum* and *TMV-N. benthamiana* systems (1,5). In this study, CMV was detected only in the external phloem in plants grown at 24°C, but was in both the external and the internal phloem in plants grown at 36°C (Fig. 6). These findings suggest that the inhibition of systemic infection by CMV in *T. expansa* grown at 24°C is due to the inhibition of viral transport between the external and the internal phloem within the veinal cells. Andrianifahanana et al. (1) and Cheng et al. (5) reported that the viruses they studied may have been transported from external to internal phloem in the stem at or below the inoculated leaves. Although the specific sites inhibiting CMV movement in *T. expansa* grown at 24°C have not yet been identified, CMV appears to be transported from the external to the internal phloem through the xylem parenchyma at least in the petioles of the inoculated leaves of plants grown at 36°C (Fig. 6). First- to third-order veins have both external and internal phloem, but minor veins have only external phloem in *T. expansa*. The virus cannot move from external phloem to internal phloem in leaf blade of the inoculated leaf except in major veins. Viral transportation from the external to the internal phloem could have occurred in the major veins and petiole of the inoculated leaf as well as in the stem.

Plant growth under high temperature conditions from 24 to 36 hpi was required for CMV to establish systemic infection (Fig. 4). Furthermore, CMV was transported from the stem into the petioles of the upper leaves at 24 to 36 hpi in plants grown at 36°C (Fig. 5). These findings confirm that there is another barrier against CMV movement after viral entry into the phloem pathway. The barrier is probably at the interface between the external and the internal phloem, as discussed previously. It should be noted that CMV was detected in the petiole of the inoculated leaf by 24 hpi but not in the petiole of the upper leaf until 36 hpi (Fig. 4), in spite of the observation that long-distance movement of plant viruses occurs at a rate in the centimeter per hour range (16). The additional 12 h necessary for CMV accumulation in the upper leaf petiole might be required for CMV to move cell-to-cell from the external to the internal phloem through major vein or stem xylem parenchyma cells at 36°C. CMV signals were detected not only in the external and the internal phloem but also in the xylem parenchyma cells at 36°C (Fig. 6). Cell-to-cell movement occurs at the rate of micrometer per hour (16), and this rate of movement is compatible with the extended time required for accumulation in the upper petioles. Increasing temperature should affect viral movement in the interface of external phloem-xylem parenchyma rather than that of xylem parenchyma-xylem parenchyma or xylem parenchyma-internal phloem, because the CMV in the petioles of the inoculated leaves was localized only in the external phloem at 24°C (Fig. 6).

There are other factors that may affect the systemic accumulation of CMV in *T. expansa*. One is the altered development and growth of vascular tissues in plants grown at 36°C versus 24°C (Fig. 6). The second is the potential inhibition of unloading of virus from the external phloem into the surrounding cells that may be due to a physical barrier such as callose deposition (17,35).

From these results, we conclude that the movement of pepo-CMV is inhibited after entry into the phloem at 24°C in *T. ex-*

Fig. 6. Immuno-histochemical analysis of *Cucurbit mosaic virus* (CMV) distribution in the petioles of inoculated leaves of *Tetragonia expansa* grown at **A**, 36°C and **B**, 24°C. Phloem tissue in the petioles is labeled as external phloem (EP) or internal phloem (IP) on either side of the xylem. **A**, CMV was detected in EP, xylem parenchyma (XP), and IP at 36°C, but **B**, was only detected in limited cells in EP at 24°C (indicated by arrowheads). V: vessel. Bars = 50 μ m.

pansa, which may be caused by the inhibition of viral transport from external to internal phloem. These results should provide important insight into further exploration of not only the temperature-sensitive resistance mechanisms of *T. expansa* against CMV infection but also the tracking of plant viruses in systemic infections. CMV strains that overcome the inhibition at 24°C would provide further information into viral-host mutuality.

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