Resistance of *Capsicum annuum* ‘Avelar’ to pepper mottle potyvirus and alleviation of this resistance by co-infection with cucumber mosaic cucumovirus are associated with virus movement

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*Capsicum annuum* cv. Avelar plants resist systemic infection by the Florida isolate of pepper mottle potyvirus (PepMoV-FL). Immuno-tissue blot analysis for detection of PepMoV-FL infection in selected stem segments revealed that virus moved down the stem in external phloem, and, over time, accumulated to detectable levels throughout stem sections (appearing to accumulate in external and internal phloem) taken from below the inoculated leaf. At 21 days post-inoculation, PepMoV-FL was detected in stem segments one or two internodes above the inoculated leaf; however, no virus was observed in internal phloem in stem segments beyond these internodes. In contrast to these observations, PepMoV-FL was detected in the internal phloem of all internodes of the stem located above the inoculated leaf, with subsequent movement into non-inoculated leaves, in Avelar plants co-infected with PepMoV-FL and cucumber mosaic cucumovirus (CMV-KM). No apparent enhancement of PepMoV-FL accumulation occurred in protoplasts inoculated with PepMoV-FL alone versus a mixed inoculum of PepMoV-FL and CMV-KM. These findings confirm earlier observations that potyvirus movement up the stem of *Capsicum* species occurs via internal phloem. It is also shown that PepMoV-FL does not accumulate to detectable levels in internal phloem in the stem of Avelar plants, thereby limiting its movement to within the inoculated leaf and lower portions of the stem; however, co-infection of Avelar plants with CMV-KM alleviates this restricted movement, allowing PepMoV-FL to invade young tissues systemically.

Introduction

Virus infection of a plant host follows a characteristic pattern of processes that must be carried out for development of a successful systemic infection. In addition to processes that involve the synthesis of virus materials, e.g. translation of genes, replication of nucleic acid and virion formation, virus must move from cell to cell, enter the vasculature (in most cases the phloem) and move to distant locales within the plant, where it must exit the vasculature and again move from cell to cell among non-vascular tissues. These processes have been reviewed extensively in recent years (Lucas & Gilbertson, 1994; Carrington et al., 1996; Gilbertson & Lucas, 1996; Séron & Haenni, 1996; Nelson & van Bel, 1998).

The plant virus-infection process could be viewed as three distinct sets of processes, i.e. those associated with replication that occur in individual cells, those associated with non-vascular cell-to-cell movement and those associated with phloem-dependent movement. The ability of a plant to resist virus infection typically is directed at one of these sets of processes. Some forms of resistance have an effect on virus accumulation at the cellular level (Maule et al., 1980; Kiefer et al., 1984; Adams et al., 1986; Fraser, 1990; Deom et al., 1997; Murphy et al., 1998). A more common form of resistance, however, is the ability of a plant to restrict virus movement, either from cell to cell (Motoyoshi & Oshima, 1977; Gibb et al., 1989; Arroyo et al., 1996), localization within the inoculated leaf (Lei & Agrios, 1986; Dufour et al., 1989; Nono-Womdin et al., 1991) or limited systemic spread (Dufour et al., 1989; Law et al., 1989; Goodrick et al., 1991; Nono-Womdin et al., 1991; Simon et al., 1992; Wilson & Jones, 1992; Nelson et al., 1993).
Murphy & Kyle, 1995; Schaad & Carrington, 1996; Derrick & Barker, 1997).

Capsicum annuum L. cv. Avelar was shown to have a monogenic, recessively inherited factor for resistance to pepper mottle potyvirus (PepMoV) (Zitter & Cook, 1973), which was recently assigned the symbol Por3 (Murphy et al., 1998). Avelar plants (and those of the C. annuum variety ‘Delray Bell’, which is derived from Avelar) inoculated with the Florida isolate of PepMoV (PepMoV-FL) were reported to remain symptomless for approximately 4 weeks before the development of a mild mottle symptom on inoculated and old, non-inoculated leaves (Zitter & Cook, 1973). A more detailed analysis of PepMoV-FL infection in Avelar plants showed that virus accumulated in inoculated leaves and moved into the stem but did not move up the stem to invade young tissues (Murphy & Kyle, 1995). Interestingly, the restricted movement of PepMoV-FL in Avelar plants was alleviated when plants were co-infected with PepMoV-FL and either cucumber mosaic cucumovirus (CMV) (Murphy & Kyle, 1995) or tobacco mosaic tobamovirus (Pieczarka & Zitter, 1981).

We were interested in understanding the nature of the resistance of Avelar to PepMoV-FL. However, such studies could not be conducted without first understanding the normal pathway of movement of PepMoV-FL in susceptible C. annuum plants (Andrianifahanana et al., 1997). In the susceptible PepMoV-FL–Capsicum interaction, virus was shown to move in a source-to-sink pattern similar to that of photoassimilates and as described for most virus (viroid)–host systems (Samuel, 1934; Bennett, 1940; Palukaitis, 1987; Turgeon, 1989; Leisner et al., 1992; Más & Pallás, 1996). Of particular interest was the observation that PepMoV-FL movement through the stem of Capsicum species followed a downward pattern in external phloem, with movement up the stem to young tissues occurring via internal phloem (Andrianifahanana et al., 1997). Our understanding of PepMoV-FL movement in susceptible hosts was used to determine the nature of the resistance of Avelar to PepMoV-FL and the basis for its alleviation of restricted movement when Avelar plants are co-infected with CMV.

**Methods**

- **Plant materials and growth conditions.** Seeds of C. annuum ‘Avelar’ and ‘Early Calwonder’ were derived originally from M. K. Jahn (Cornell University, Ithaca, NY, USA). Avelar seed used in this study was derived from a single plant shown to express the highest level of resistance to PepMoV-FL under greenhouse conditions (J. F. Murphy, unpublished data). Seeds were surface-sterilized (Murphy & Kyle, 1995) prior to being transplanted to 10 × 10 cm pots containing Pro-Mix soilless potting medium (Premier Peat, Rivière-du-Loup, Québec, Canada). Plants were grown in a temperature-controlled greenhouse (mean temperatures of 28 °C day/21 °C night) with supplemental metal-halide lighting used for 14 h a day from September to April.

- **Virus isolates and inoculations.** PepMoV-FL was provided by T. A. Zitter (Cornell University, Ithaca, NY, USA), maintained in light used for 14 h a day from September to April. Temperatures of 28 °C were grown in a temperature-controlled greenhouse (mean temperatures of 28 °C day/21 °C night) with supplemental metal-halide lighting used for 14 h a day from September to April.

**Fig. 1.** A Capsicum annuum plant showing the locations along the stem that were representative of internodes used for generation of tissue blots presented as columns 1–4 in Figs 2 and 3.
Results

Phloem-dependent movement of PepMoV-FL in Avelar stems

PepMoV-FL CP was typically not detected in the stem of Avelar plants at 7 days p.i. (Fig. 2, A1–A4); however, when detection did occur, it was localized to the external phloem in the internode below inoculated leaf 1 (data not shown). By 9 days p.i., PepMoV-FL CP was routinely detected at the internode below inoculated leaf 1 (Fig. 2, B1) but no viral CP was detected in any internode located above inoculated leaf 1 (Fig. 2, B2–B4). This trend continued at 12 days p.i., with a notable increase in PepMoV-FL CP accumulation in the internode below the inoculated leaf, particularly internal to the vascular ring, i.e. in the pith (Fig. 2, C1). A small amount of PepMoV-FL CP was detected in external phloem in the first internode above the inoculated leaf (Fig. 2, C2), but not in the internal phloem in any of the internodes located above the inoculated leaf (Fig. 2, C2–C4). By 21 days p.i., PepMoV-FL CP was detected in the internode below inoculated leaf 1 and in the first two internodes above inoculated leaf 1 (Fig. 2, D1–D3, respectively), but not in any successive internode (e.g. internode between leaves 10 and 11, as shown in Fig. 2, D4). In each of the stem segments shown to contain PepMoV-FL CP at 21 days p.i., accumulation appeared to occur in both external and internal phloem.

Phloem-dependent movement of PepMoV-FL in Avelar stems co-infected with CMV

PepMoV-FL CP was detected in all stem sections of Avelar plants co-infected with CMV by 5 days p.i. (Fig. 3, A1–A4). This is in contrast to the restricted movement of PepMoV-FL in singly infected Avelar plants, in which virus was not detected beyond the two internodes immediately above the inoculated leaf at 21 days p.i. (Fig. 2). In PepMoV+CMV-infected Avelar plants at 5 days p.i., PepMoV-FL CP was detected in external and internal phloem in the internode below inoculated leaf 1 (Fig. 3, A1) and in the internal phloem in all internodes above the inoculated leaf (Fig. 3, A2–A4; arrowheads indicate examples of the location of PepMoV-FL CP labelling in internal phloem). The amount of PepMoV-FL CP and its distribution within the stem increased progressively at 7 (Fig. 3, B1–B4), 9 (Fig. 3, C1–C4) and 12 (Fig. 3, D1–D4) days p.i., with almost complete infection across the width of stem sections and including essentially all tissue types by 12 days p.i. The pattern of movement of PepMoV-FL in co-infected Avelar plants, i.e. movement in external and internal phloem with movement up the stem of the plant occurring via internal phloem, followed the ‘normal’ pattern of movement observed for potyviruses through the stem of susceptible Capsicum hosts (Andrianifahanana et al., 1997). PepMoV-FL was detected in external and internal phloem in stem segments below inoculated leaf 1 but only in internal phloem in stem segments above inoculated leaf 1. Additionally, virus accumu-
lated in an asymmetric pattern along the stem, always occurring on the side to which the inoculated leaf was attached (this would represent the left side of each tissue print). Consistent with this asymmetric pattern of accumulation within the stem is the progressive pattern of accumulation across the stem. Virus detection occurred initially in small, localized areas on the side of the stem to which the inoculated leaf was attached. Over time, the spread of the infection was observed to occur across the stem until essentially all tissues were shown to contain PepMoV-FL CP. This pattern of lateral movement tended to occur progressively from lower stem segments to upper stem segments. Whether this represents a lateral cell-to-cell type of movement was not determined, although it occurred consistently with PepMoV-FL and CMV in Avelar as well as with PepMoV-FL in Early Calwonder (Andrianifahanana et al., 1997).

CMV-KM accumulation and movement through the stem of Avelar plants followed a similar asymmetric pattern to that observed with potyviruses (Fig. 3, E1–E4). However, the delineation between CMV-KM CP accumulation in external versus internal phloem, as observed for the potyviruses, was not observed clearly in any of the experiments. The indiscriminate accumulation of CMV CP in both external and internal phloem occurs with CMV in all other *C. annuum* varieties and *Capsicum* species evaluated to date (J. F. Murphy, unpublished data). In PepMoV + CMV-infected Avelar plants, CMV-KM CP was detected over the entire length of the stem by 5 days p.i. (Fig. 3, E1–E4), with a dramatic increase in the amount of CMV-KM CP detected by 7 days p.i. (Fig. 3, F1–F4). It should be noted that the extent and timing of CMV-KM accumulation and movement in Avelar stems was the same, regardless of whether plants were infected with CMV-KM alone or in combination with PepMoV-FL (data not shown).

In PepMoV + CMV-infected Avelar plants, PepMoV-FL CP was detected in the stem at earlier times p.i. than in Avelar plants infected with PepMoV-FL alone. Furthermore, the amount of PepMoV-FL and the extent of its movement throughout the stem was greater in Avelar plants infected with PepMoV + CMV than in Avelar plants infected with PepMoV-FL alone. However, the time p.i. at which PepMoV-FL CP was detected and the extent of PepMoV-FL accumulation throughout the stem clearly lagged behind those of CMV-KM in co-infected plants. This is illustrated in the stem blots taken from PepMoV + CMV-infected Avelar plants at 5 and 7 days...
Bioassay for infectious virus in stem segments

All stem segments from each Early Calwonder plant (susceptible host) inoculated either with PepMoV-FL alone, CMV-KM alone or the combined inoculum of PepMoV + CMV contained the respective virus or viruses, as revealed by successful transfer to young Early Calwonder plants. In Avelar plants inoculated with PepMoV-FL alone, infectious PepMoV-FL was transferred to Early Calwonder plants from the hypocotyl, from each internode below inoculated leaf 3 and (for two of seven plants) from the internode immediately above the inoculated leaf (internode 3/4). No PepMoV-FL was detected from any internode segment above leaf 4 in Avelar plants inoculated with PepMoV-FL alone. In contrast, PepMoV-FL was detected in each internode of each Avelar plant that was inoculated with the combined inoculum, PepMoV + CMV. Likewise, CMV-KM was detected in all internodes of each Avelar plant inoculated with either CMV-KM alone or PepMoV + CMV.

Accumulation of PepMoV-FL and CMV CP in protoplasts

Avelar mesophyll protoplasts were isolated and inoculated with PepMoV-FL, CMV-KM or PepMoV + CMV, in an attempt to determine whether PepMoV-FL accumulation was enhanced at the cellular level by co-infection with CMV-KM. PepMoV-FL CP accumulation in Avelar protoplasts was similar, regardless of whether inoculum consisted of PepMoV-FL alone (Fig. 4; PepMoV, lanes 1 and 2) or the combined inoculum of PepMoV + CMV (Fig. 4, lanes 3 and 4). CMV-KM CP accumulation was also unaffected by whether inoculum consisted of CMV-KM alone (Fig. 4; CMV, lanes 1 and 2) or PepMoV + CMV (Fig. 4, lanes 3 and 4). This experiment was performed four times, each time using different sources of inocula and freshly isolated Avelar protoplasts, with similar results obtained between experiments.

Discussion

In Avelar plants, PepMoV-FL was shown to accumulate in inoculated leaves and lower portions of the stem with no detectable movement to or accumulation in young, non-inoculated tissues (Murphy & Kyle, 1995; M. N. Guerini & J. F. Murphy, unpublished data). A more detailed analysis of PepMoV-FL movement through the stem of Avelar plants has revealed that virus did move into the stem, where it accumulated in external phloem in stem segments taken from locations below the inoculated leaf (Fig. 2). At later times p.i., PepMoV-FL accumulated to relatively high levels in external and internal portions of stem segments positioned below the inoculated leaf and even in internodes one or two positions above the inoculated leaf. Based on anatomical studies of the Capsicum stem, PepMoV-FL accumulated in areas of the stem in which internal phloem was located. However, there was no sign of the rapid movement of virus (based on accumulation and detection of PepMoV-FL CP by immuno-tissue blot analysis or by bioassay for infectious virus) up the stem to young tissues within the internal phloem. These observations suggest that the resistance of Avelar to PepMoV-FL is in the form of a blockage of entrance into the internal phloem or into those internal phloem bundles that translocate photosynthates up the stem to young tissues. The occurrence of PepMoV-FL in stem segments above the inoculated leaf by 21 days p.i. may have resulted from cell-to-cell movement. In contrast to these observations, the pattern of PepMoV-FL movement and extent of accumulation in Avelar plants co-infected with PepMoV-FL and CMV-KM was similar to that observed for PepMoV-FL in the susceptible host Early Calwonder (Andrianifahanana et al., 1997).

The precise route taken by virus for entrance into internal phloem of the stem has not been determined. On the basis of previous reports (Bonnemain, 1969; Andrianifahanana et al., 1997) and data presented here, two pathways can be hypothesized. One pathway involves entrance of virus into external phloem of the stem directly from the external phloem of the petiole of the infected source leaf. Upon entrance into the stem, virus would move down the stem via external phloem until reaching a location(s) containing channel-like connections between external and internal phloem. Once in the internal phloem, virus would move rapidly up the stem to developing tissues. A second pathway would involve entrance of virus into the stem by way of both external and internal phloem from the petiole of the infected source leaf. In this scenario, virus entering the stem in the external phloem would be transported down the stem, whereas virus moving through internal phloem of the petiole would enter directly into internal
phloem of the stem. There would be an initial phase of downward movement in the stem until a location was attained where there were anastomoses between internal phloem bundles, e.g. at a leaf node. Virus would then enter neighbouring internal phloem bundles that transport photoassimilates (and virus) up the stem to leaves that act as sinks. The ring-like pattern of PepMoV-FL accumulation in internal phloem observed in tissue prints from stem segments above the inoculated leaf (e.g. Fig. 3, B3, B4 and C3 and as described by Andrianifahanana et al., 1997) supports the suggestion of anastomoses between internal phloem bundles. In the case of PepMoV-FL in Avelar plants (and CMV; Dufour et al., 1989), we hypothesize that virus enters external phloem but is not able to enter internal phloem in the inoculated leaf. As a result, virus moves through the petiole in the external phloem and continues into the stem, where it is transported down the stem. Since PepMoV-FL is not able to enter internal phloem within the inoculated leaf, it does not gain access to the appropriate internal phloem of the stem and thus, does not move up the stem to young tissues. In Avelar plants co-infected with PepMoV-FL and CMV-KM, CMV-KM somehow allows PepMoV-FL to circumvent the blockage of entrance into internal phloem, thereby resulting in movement into the stem and up the stem to young tissues via internal phloem. We have not determined whether PepMoV-FL occurs in external and internal phloem in the petiole of susceptible varieties and Avelar plants, as illustrated for CMV in Capsicum (Dufour et al., 1989), although this information will be critical in our understanding of the resistance of Avelar.

Plant resistance to virus infection expressed in the form of restricted systemic movement of the virus has been described for many other virus-host systems (Lei & Agris, 1986; Dufour et al., 1989; Law et al., 1989; Goodrick et al., 1991; Nono-Womdin et al., 1991; Simon et al., 1992; Wilson & Jones, 1992; Nelson et al., 1993; Schaad & Carrington, 1996; Derrick & Barker, 1997). In a few cases, the general location or types of tissues associated with the blockage have been identified, though most appear to involve an inability of the virus to enter the phloem or exit the phloem or combinations thereof. Goodrick et al. (1991) showed that cowpea chlorotic mottle bromovirus was able to move from cell to cell in the inoculated leaf in the resistant soybean genotype PI 346304 but was rarely detected within the vascular tissue. Schaad & Carrington (1996) showed that tobacco etch potyvirus (TEV) was restricted in its ability to move systemically in the tobacco line V20 even though virus was detected in phloem parenchyma and companion cells. These authors suggested the occurrence of a blockage in the ability of TEV to enter into or exit from sieve elements. Resistance responses more similar to that of Avelar to PepMoV-FL were described by Dufour et al. (1989), Simon et al. (1992) and Derrick & Barker (1997). In each case, virus moved into the stem of infected plants but was restricted in its ability to move to young tissues. Turnip crinkle carmovirus moved throughout and accumulated in the inoculated leaf of Arabidopsis thaliana ecotype Dijon plants as well as in the leaf on the opposite side of the stem; however, accumulation in vascular tissues and younger leaves was restricted, relative to a susceptible control (Simon et al., 1992). Derrick & Barker (1997) showed that potato leafroll luteovirus (PLRV) accumulated in external and internal phloem of susceptible plants, but in resistant potato lines, resistance was expressed as an exclusion of PLRV from external phloem bundles in the stem. These authors suggested that, in resistant plants, movement of PLRV from sieve elements to companion cells was dramatically impaired, resulting in a reduction in the number of external phloem cells that became infected. Dufour et al. (1989) identified a phloem-associated resistance in Capsicum against CMV. In their study, CMV was detected in internal and external phloem of the petiole in the susceptible variety but only in the external phloem of the resistant variety. Moreover, CMV infection spread into the stem of the resistant plant, again being localized to external phloem, and in a similar manner to our observations with PepMoV-FL in Avelar, CMV remained in the lower portions of the stem (Dufour et al., 1989).

On the basis of our understanding of directional flow of photoassimilates and virus in Capsicum species (Bonnemain, 1969; Andrianifahanana et al., 1997), movement down the stem occurs via external phloem while movement up the stem occurs via internal phloem. Taken together, the evidence strongly suggests that restriction of virus (i.e. CMV or potyvirus) to external phloem in Capsicum species results in a limited systemic infection, whereby virus is able to accumulate in and move through the petiole of the inoculated leaf and into the stem, where it accumulates in lower portions of the stem. If virus does not enter the appropriate internal phloem, the location or pathway of which has yet to be determined, then rapid movement up the stem via internal phloem does not occur.

The likely explanation for the resistance observed in Avelar [and perhaps that described by Dufour et al. (1989)] is that PepMoV-FL is unable to enter, accumulate or move or combinations thereof in the internal phloem of the stem. An alternative explanation could be associated with virus particle stability within the phloem. Mutations of the TEV coat protein were shown to affect long-distance movement (Dolja et al., 1994, 1995) and Fuentes & Hamilton (1993) showed that southern bean mosaic sobemovirus was unable to move long distances in a resistant host because the virus particles were not stable in vascular tissues. Since PepMoV-FL does move systemically via internal phloem in Avelar plants co-infected with CMV-KM, and the PepMoV-FL that occurs in the upper portions of co-infected stems is infectious, virus particle instability is an unlikely explanation.

Mixed infections, i.e. infection of a plant by more than one type of virus, occur commonly in nature and may result in a range of effects on the host as well as on the levels of accumulation and degrees of movement of either of the viruses involved. Of particular interest to our work are those mixed
infections in which the restricted ability of one virus to move is alleviated by co-infection with another virus (Hamilton & Nichols, 1977; Pieczarka & Zitter, 1981; Carr & Kim, 1983; Barker, 1987; Malyshenko et al., 1989; Fuentes & Hamilton, 1991; Murphy & Kyle, 1995). Our results show that in Avelar plants co-infected with PepMoV-FL and CMV-KM, PepMoV-FL is able to enter, accumulate in and move through internal phloem, thereby allowing the virus to invade young tissues systemically. Furthermore, the appearance of PepMoV-FL in the stem of Avelar occurred sooner in co-infected plants than in plants infected with PepMoV-FL alone, although PepMoV-FL detection always lagged behind that of CMV-KM. There was no apparent difference in PepMoV-FL accumulation in Avelar protoplasts inoculated with either PepMoV-FL alone or as a mixed inoculum with CMV-KM. This suggests that CMV-KM did not enhance PepMoV-FL accumulation at the cellular level, but rather compensated PepMoV-FL in its inability to enter internal phloem.

References


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