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# Effects of Host Plant Development and Genetic Determinants on the Long-Distance Movement of Cauliflower Mosaic Virus in Arabidopsis

**Scott M. Leisner,[a] Robert Turgeon,[b] and Stephen H. Howell[a,1]**

[a] Boyce Thompson Institute, Tower Road, Ithaca, New York 14853
[b] Section of Plant Biology, Cornell University, Ithaca, New York 14853

**During systemic infections, viruses move long distances through the plant vascular system. The long-distance movement of cauliflower mosaic virus (CaMV) in Arabidopsis has been examined using a whole plant in situ hybridization technique called plant skeleton hybridization. CaMV moves long distance through the phloem largely following the flow of photoassimilates from source to sink leaves. During the course of plant development, sink–source relationships change and the region of the plant that CaMV can invade is progressively reduced. In Arabidopsis, we have found that conditions that influence the rate of plant development dramatically impact the long-distance movement of CaMV, because under normal conditions the rate of plant development is closely matched to the kinetics of virus movement. Ecotypes and mutants of Arabidopsis that flower early show a form of resistance to systemic CaMV infection, which we call "developmental resistance." Developmental resistance results from the fact that the rosette leaves mature early in the life of an early flowering plant and become inaccessible to virus. On the other hand, if the development of early flowering plants is retarded by suboptimal growth conditions, inoculated plants appear more susceptible to the virus and systemic infections become more widespread. We have found that other Arabidopsis ecotypes, such as Enkheim-2 (En-2), show another form of resistance to virus movement that is not based on developmental or growth conditions. The virus resistance in ecotype En-2 is largely conditioned by a dominant trait at a single locus.**

## INTRODUCTION

During the course of viral systemic infection in plants, viruses replicate and move short distances from cell to cell and long distances through the vascular system (Agrios, 1988). Unlike animal viruses that usually move extracellularly from cell to cell by budding or lysis and reinfection, plant viruses move intracellularly from cell to cell through cytoplasmic bridges or plasmodesmata. Virus movement through plasmodesmata requires viral-encoded movement proteins, the best known of which is the 30K "movement" protein of tobacco mosaic virus (TMV) (reviewed by Hull, 1989). The 30K protein is thought to dilate the plasmodesmata and can act in *trans* to rescue viruses defective in movement functions (Deom et al., 1987). The form in which viral nucleic acids move from cell to cell is still an open question. It has been argued, in the case of TMV, that the infectious entity that moves from cell to cell is a ribonuclear protein complex (Citovsky et al., 1990, 1992). This is based on the observation that the 30K movement protein of TMV cooperatively binds RNA and forms a thin rod capable of moving through modified plasmodesmata.

For spherical caulimoviruses, such as cauliflower mosaic virus (CaMV), there are convincing electron microphotographs that show virus particles in the plasmodesmata between infected cells (Kitajima and Lauritis, 1969). It is not known whether these images demonstrate that intact virus particles move from cell to cell or whether they simply represent structural abnormalities found late in infection. Despite these observations, there has been speculation that the infectious entity that moves from cell to cell in CaMV-infected plants is also a ribonuclear–protein complex, as it is in TMV-infected plants (Citovsky et al., 1991). This argument is based on the observations that gene I in CaMV encodes a protein, related in sequence to the TMV 30K protein, that is found in the cell wall (Albrecht et al., 1988; Linstead et al., 1988), and is capable of binding RNA (Citovsky et al., 1991).

Less is known about long-distance virus movement: how viruses enter, move through, and exit the vascular system. It is likely that both host and viral functions required for long-distance movement are different from those needed for cell-to-cell movement (Hull, 1989; Maule, 1991). First, within minor veins the plasmodesmata connecting phloem parenchyma with bundle sheath cells and these two cell types to companion cells differ from those interconnecting other cell types (Beebe and Evert, 1992; Ding et al., 1992). Therefore, it is possible that viruses must utilize different mechanisms to pass through

[1] To whom correspondence should be addressed.

these channels into the phloem. Second, viral genes required for long-distance movement appear to be different from those required for cell-to-cell movement. Mutations in either the coat protein or the assembly origin of TMV greatly delay or abolish viral long-distance movement while allowing cell-to-cell movement to proceed normally in the inoculated leaf (Dawson et al., 1988; Saito et al., 1990).

Third, certain phloem-limited viruses can move long distances in the vasculature but cannot move out into leaf mesophyll cells. Interestingly, some of these phloem-limited viruses can move from mesophyll cell to mesophyll cell with the help of nonphloem-limited viruses in mixed infections (Barker, 1987; Atabekov and Taliansky, 1990). Also, some of these viruses can replicate in mesophyll protoplasts of the same host plants in which the virus is limited to the phloem (Barker and Harrison, 1982; Barker, 1987). These observations suggest that certain phloem-limited viruses do not lack the ability to replicate in nonvascular tissues; however, they are unable to invade those tissues. Finally, virus resistance genes in plants have been described that block the long-distance movement of viruses while they permit viruses to replicate and move from cell to cell in inoculated leaves (Kuhn et al., 1981; Lei and Agrios, 1986; Dufour et al., 1989; Law et al., 1989; Goodrick et al., 1991).

Once viruses have invaded the vascular system in susceptible host plants, they move in prescribed pathways preferentially following certain routes in the course of an infection (Samuel, 1934). CaMV, which moves systemically through phloem channels in plants such as turnip (Leisner et al., 1992), is apparently swept along with the flow of photoassimilates from source leaves to sink leaves. Therefore, not all parts of a plant are accessible to viruses during systemic infection, but only those parts into which photoassimilates flow. During plant development, sink–source relations change and so do the patterns of virus movement (Leisner et al., 1992). Young leaves import photoassimilates, whereas mature leaves export photoassimilates. In systemic infections, young leaves import viruses from inoculated leaves, whereas mature leaves do not. However, the parts of the plant accessible to viruses are even more limited than those accessible to photoassimilates because young leaves stop importing viruses before they stop importing photoassimilates (Leisner et al., 1992). Thus, in plants with determinate growth patterns, the region of the plant accessible to systemic virus infection is continuously reduced during development.

In this study, we have found that developmental changes have a dramatic impact on the long-distance movement of CaMV in Arabidopsis because, under normal conditions, the rate of plant development is closely matched to the kinetics of virus movement. Early flowering Arabidopsis ecotypes appear resistant to systemic CaMV infection because most of the vegetative parts of the plant mature early in the lifetime of the plant and become inaccessible to virus even when plants are inoculated at early stages in plant development. In addition, certain Arabidopsis ecotypes show other forms of resistance to virus movement that are not directly based on developmental constraints. In one ecotype, Enkheim-2 (En-2), resistance to virus movement is largely conferred by a single, dominant trait.

## RESULTS

### Movement of CaMV in Infected Arabidopsis Plants

To trace the movement of CaMV in Arabidopsis, we expanded the scale of an in situ hybridization technique, called the leaf skeleton hybridization procedure developed by Melcher and colleagues (1981, 1989), to entire Arabidopsis plants. In this "plant skeleton hybridization" technique, labeled viral DNA probe is hybridized to whole Arabidopsis plants that have been appropriately fixed and prepared for hybridization. DNA in the specimen that hybridizes with the probe most likely represents virions as well as other nonencapsidated, viral DNA forms because the procedure used in preparing the whole plant skeletons releases viral DNA from virions. In virus–plant combinations where CaMV produces visible symptoms, the pattern of symptoms closely corresponds to the pattern of hybridization. In Figure 1 where the standard Arabidopsis ecotype, Columbia (Col-0), was inoculated with CaMV isolate CM4-184, viral DNA was found throughout the plant. Virus DNA was found in roots, rosette leaves, flower stalks, and cauline leaves (flower stalk leaves) in younger, infected plants (Figure 1B), and in older, infected plants virus DNA was observed additionally in siliques or seed pods (Figure 1C). Viral DNA was not always uniformly distributed over various organs of the plant, for example, in siliques virus DNA accumulated at the tips.

The plant skeleton hybridization technique allowed us to observe the distribution of virus DNA and, hence, the pathways of virus long-distance movement throughout entire Arabidopsis plants. However, there were some limitations in using this technique. Like any other hybridization technique, there was a limit on the sensitivity of detection. Clearly, we were able to detect the virus in systemically infected leaves, but not along all channels leading from the inoculated leaf to infected leaves. For example, there were sections of flower stalks through which the virus must have passed to infect cauline leaves, and siliques that showed little evidence of hybridization (data not shown). This suggests that either the technique is too insensitive to detect the small quantities of virus present within these intervening vascular channels or that the virus had already moved through these channels and did not exit the vascular system along the way to initiate other infections. In other cases, the flower stalks themselves were systemically infected and marked by patches or plaques of hybridization that appeared to be small centers of infection associated with the vascular channels (Figures 1B and 1C).

Because Arabidopsis is a rapidly growing plant, we were interested in comparing the rate of systemic movement of CaMV to the rate of development of the plant. To examine the rate of systemic CaMV spread in Arabidopsis, we harvested plants at various times after inoculation. As shown in Figure 2, we

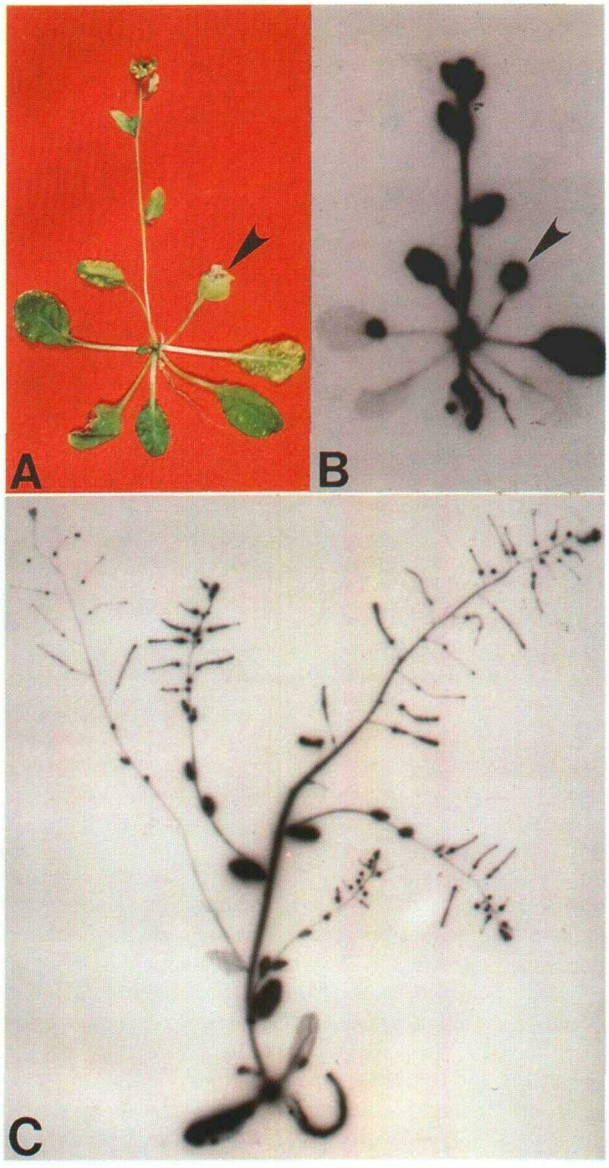


**Figure 1.** Plant Skeleton Hybridization Technique Showing Distribution Pattern of CaMV DNA in Systemically Infected Arabidopsis Plants.

Arabidopsis ecotype Columbia (Col-0) was inoculated with CaMV isolate CM4-184.

**(A)** Photograph of visible symptoms produced by CaMV on a plant harvested 27 days postinoculation. Arrow indicates inoculated leaf.

**(B)** Autoradiograph of CaMV DNA hybridization signal of the plant shown in **(A)**.

**(C)** Autoradiograph of CaMV DNA hybridization signal of a plant harvested 40 days postinoculation.

could detect a modest, localized signal in inoculated leaves only 2 days after inoculation. However, it was not until 14 days after inoculation that a hybridization signal could be detected in the petioles of the inoculated leaves. Nonetheless, by 18 days after inoculation, viral DNA could be detected in other rosette leaves in addition to the inoculated leaf. By day 22 after inoculation, virus DNA was found in the flower stalk and infected cauline leaves, and by day 26, all parts of the plant accessible to CaMV had been infected. The time from germination to bolting in the standard ecotype, Col-0, under our conditions, is 22 days. Hence, the systemic movement of CaMV is closely matched to the vegetative growth cycle of standard Arabidopsis ecotypes.

We have reported that CaMV in turnip moves long distance through the phloem vasculature with the flow of photoassimilates (Leisner et al., 1992). Assuming that this is also the case in Arabidopsis, it might be possible to predict the pathway of systemic movement by knowing certain critical features about the pattern of photoassimilate flow from the inoculated leaf. The distribution of photoassimilates was determined by labeling a source leaf with $^{14}CO_2$ and following the movement of the assimilated label. As shown in Figure 3, we found at 16 days after germination that labeled photoassimilates from source leaf 3 were transported into younger rosette leaves. (At this stage, the plant had not yet developed buds and, therefore, the flower stalk had not elongated.) However, in plants 24 days after germination, no more than trace amounts of labeled photoassimilates were imported by the (now mature) rosette leaves from source leaf 3. Nonetheless, photoassimilates moved into the flower stalk and the youngest cauline leaves. These findings led us to predict that during the course of Arabidopsis development, the rosette leaves become progressively inaccessible to the import of photoassimilates and viruses from any given source leaf.

From these considerations, we have hypothesized that there is only a small window in Arabidopsis development during which virus can systemically infect rosette leaves. This is due to the fact that Arabidopsis grows and matures rapidly compared to the kinetics of systemic CaMV infection. If this is true, then we would anticipate that systemic infection would be more widespread; that is, it would include more plant organs if the rate of plant development was retarded (without slowing the rate of virus systemic infection). We used a more rapidly developing Arabidopsis ecotype to test this prediction and grew these plants under reduced illumination conditions to retard the rate of plant growth. When plants of the Wassilewskija (Ws-0) ecotype were grown under optimal conditions (continuous illumination at 50 PAR), the plants bolted after developing four to six rosette leaves. As shown in Figure 4A, under these conditions, systemic CaMV infection as visualized by the plant skeleton hybridization technique was confined to the flower stalks and siliques and did not spread to the cauline or rosette leaves. However, if the plants were grown under suboptimal conditions (12-hr light at 50 PAR), virus was found in cauline leaves as well as in flower stalks and siliques (Figure 4B). Under even less optimal conditions (8-hr light at 40 PAR), the plants flowered late and virus appeared in rosette leaves as well as on cauline leaves, flower stalks, and siliques (Figure 4C).

Another way to test the prediction that virus long-distance movement should become progressively localized to the upper

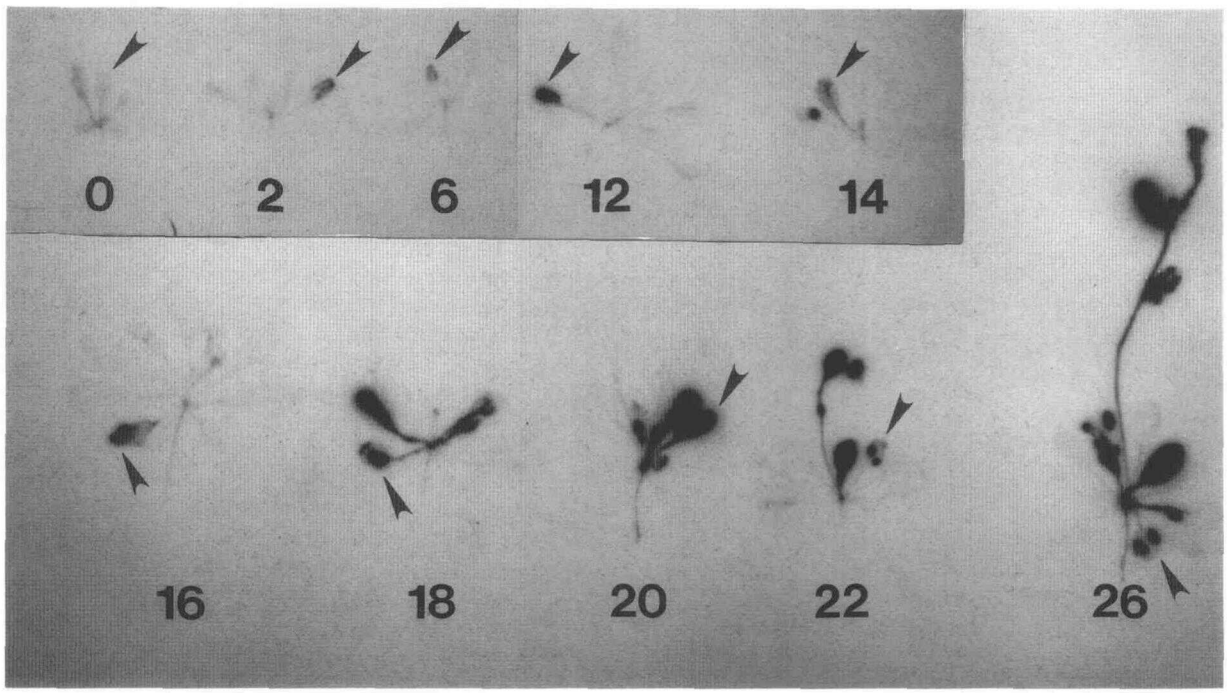


**Figure 2.** Time Course of Spread of CaMV in Arabidopsis.

Two weeks after germination, Arabidopsis plants (Columbia ecotype) were inoculated with CaMV CM4-184 on leaf 3. Plants were harvested at the number of days postinoculation indicated, and prepared for plant skeleton hybridization. Arrows indicate inoculated leaves.

part of the plant during development is to inoculate plants at different times during development. In Figure 5, we show the effect of development at the time of inoculation on the distribution of virus. For plants inoculated with CaMV at 9 days after germination, viral DNA is present in many rosette leaves, as determined by plant skeleton hybridization (24 days later). When plants were inoculated 11 days after germination, viral DNA was observed in rosette leaves, cauline leaves, and the flower stalk. When plants were inoculated 15 days after germination, viral DNA was also detected in rosette leaves as well as in cauline leaves and flower stalks, but fewer rosette leaves were invaded than in the 11-day plants. For plants inoculated 17 days after germination, although viral DNA was found in the flower stalk and cauline leaves, viral DNA was not detected in rosette leaves (other than the inoculated ones). For plants inoculated with CaMV 27 days after germination, virus was found only in flower stalks and siliques, but not in cauline or rosette leaves (except for the inoculated leaf). This shows that as plants mature, the region accessible to systemic virus infection progressively decreases.

Consistent with these observations, we found in other studies that when a whole range of Arabidopsis ecotypes was inoculated with CaMV, the earlier flowering ecotypes generally appeared more resistant to virus infection (Leisner and Howell, 1992). Figure 6 illustrates the effects of the rate of plant development for the different ecotypes which were all harvested and prepared for hybridization at the same time after germination (49 days). In an earlier flowering ecotype, such as Ws-0 (15.5 days to bolting), CaMV DNA was confined to the upper parts of the plant, the flower stalks and siliques. In later flowering ecotypes, Frankfurt-2 (Fr-2, 41 days to bolting) and Finland-3 (Fl-3, 106 days to bolting), symptoms were more widespread over different organ types including rosette leaves. Hence, the early flowering ecotypes appear to be resistant to the virus because the rosette leaves have matured and are largely inaccessible to the virus at a time when the infection becomes systemic. Plants of the Rschew-4 (Rsch-4) ecotype develop at a moderate rate; they are exceptions to the rule because they exhibit viral DNA in only siliques, flower stalks, and cauline leaves but not rosette leaves. However, the plant shows the virus distribution pattern of earlier flowering ecotypes, that is, ecotypes that flower later than ecotypes such as Ws-0 but earlier than Col-0.

Because Arabidopsis ecotypes are genetically different, it is possible that many factors contribute to the movement and apparent resistance of the early flowering plants. Therefore, we tested whether a single gene mutation that influences the time to flowering in Arabidopsis would have similar effects. The early flowering mutant studied was the terminal flower (*tfl1-1*) mutant of Shannon and Meeks-Wagner (1991). When the *tfl1-1* mutant was inoculated at the same stage in development as its wild-type counterpart, as shown in Figure 7, we found that visible systemic symptoms were confined to the flower stalk and cauline leaves in the mutant. We have also

shown by plant skeleton hybridization that the distribution of virus corresponds to the pattern of symptoms in the mutant (data not shown). Hence, a single gene that controls the rate of development in Arabidopsis confers resistance to CaMV.

### Enkheim-2, an Arabidopsis Ecotype Resistant to Certain CaMV Isolates

From a more extensive survey of Arabidopsis ecotypes (Leisner and Howell, 1992), ecotype En-2 was resistant to CaMV even though it was not an especially early flowering ecotype (27 days to bolting). Because the En-2 ecotype did not develop visible systemic symptoms when inoculated with CaMV isolate CM4-184, we used the whole plant skeleton hybridization technique to determine the fate of the inoculated virus. As shown in Figure 8, we observed that CaMV was locally confined to the inoculated leaf and the small stem in the center of the rosette. The virus did not move to other rosette leaves or to the flower stalk and cauline leaves, as it did in the Col-0 ecotype. In the case of ecotype En-2, resistance to virus movement did not appear to be related to plant development. Whether plants of the En-2 ecotype were inoculated at the three-true-leaf stage, the earliest stage when seedlings can be easily inoculated, or at any point through the six-true-leaf stage, no systemic symptoms were observed. (After the six-true-leaf stage, it might be expected that the production of systemic

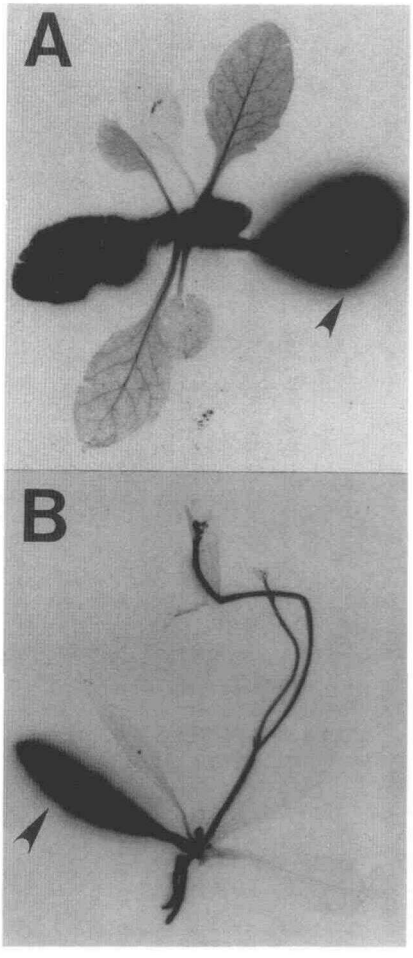


**Figure 3.** Effects of Development on the Movement of Photoassimilates in Arabidopsis.

The pattern of photoassimilate movement was examined in Arabidopsis Col-0 seedlings at two different ages. The leaves marked with arrows (leaf 3) were incubated in situ with $^{14}CO_2$ for 5 min. One hour later, the plants were prepared for autoradiography.
**(A)** Pattern of photoassimilate movement was examined in a plant 16 days after germination.
**(B)** Pattern of photoassimilate movement was examined in a plant 24 days after germination.

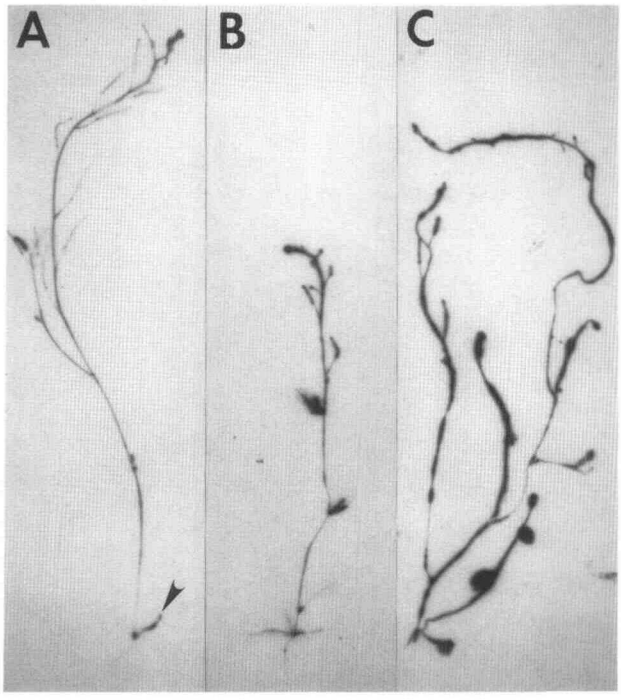


**Figure 4.** Distribution Pattern of CaMV DNA in Arabidopsis Plants Grown Under Various Illumination Conditions to Control the Rate of Plant Development.

**(A)** Plants grown under continuous illumination at 50 PAR bolted at 3 days postinoculation.
**(B)** Plants grown in 12-hr light at 50 PAR bolted at 7 days postinoculation.
**(C)** Plants grown in 8-hr light at 40 PAR bolted at 88 days postinoculation.
Arabidopsis Ws-0, a rapid bolting ecotype, in **(A)** and **(B)** was inoculated with CaMV isolate CM4-184 14 days following germination. A similar plant grown in 8-hr light **(C)** grew very slowly and was not inoculated until 24 days following germination. Plants were harvested and prepared for plant skeleton hybridization at 17 **(A)**, 34 **(B)**, and 144 **(C)** days postinoculation. Arrow indicates inoculated leaf in **(A)**. Inoculated leaves in **(B)** and **(C)** senesced before harvest.

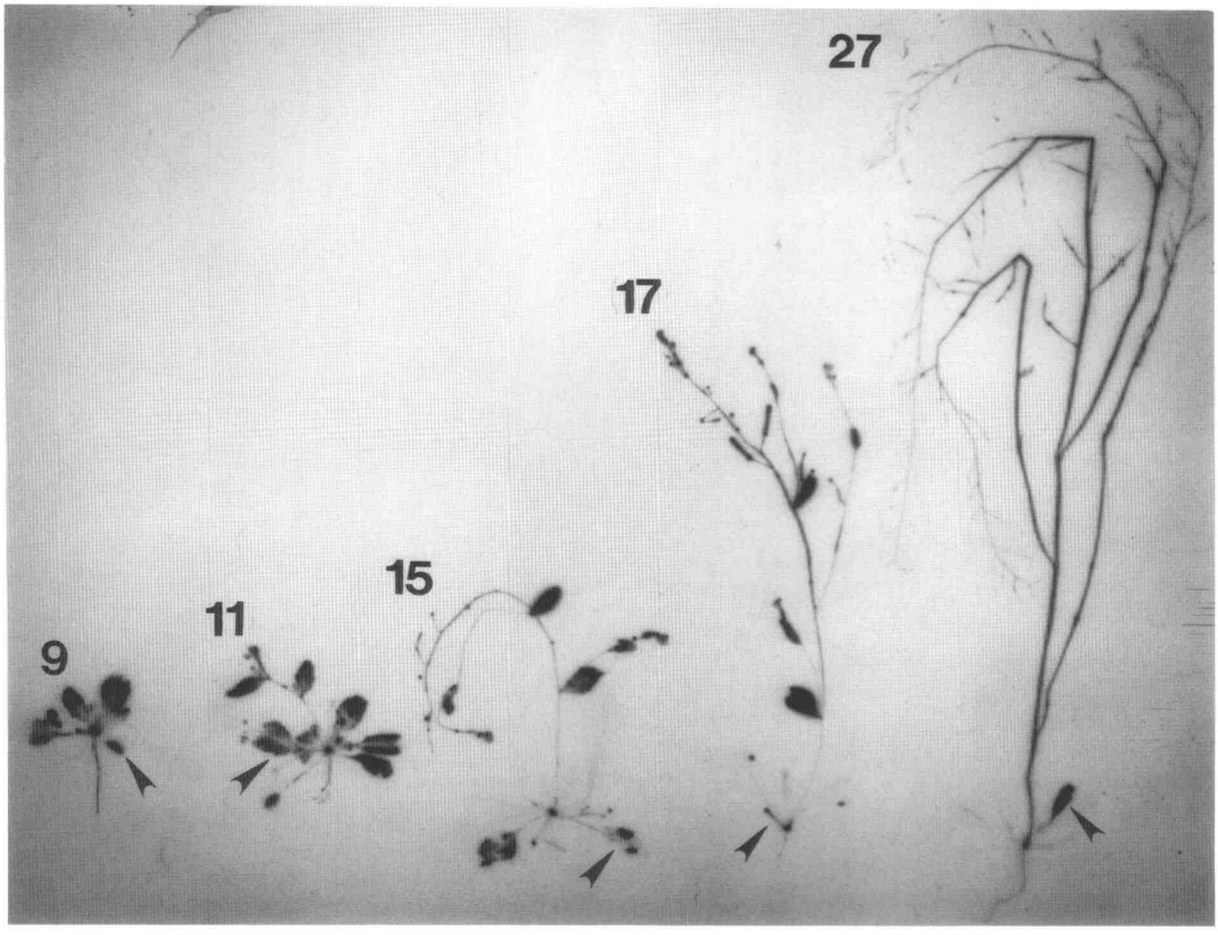


**Figure 5.** Effect of Plant Age on the Systemic Spread of CaMV.

Plants 9, 11, 15, 17, and 27 days after germination (left to right, respectively) were inoculated with CaMV CM4-184 on leaf 3. Plants were harvested at 24 days postinoculation and prepared for plant skeleton hybridization. Inoculated leaves are indicated by arrows for all plants except the 17-day plant from which the inoculated leaf fell off during processing, but the petiole from the inoculated leaf still remains and is marked by an arrow.

symptoms would be curtailed for developmental reasons even in susceptible plants.)

Because CaMV CM4-184 was limited in its systemic movement in the En-2 ecotype, we attempted to determine the genetic complexity of the plant trait preventing virus movement. To do so, we crossed ecotype En-2 with the standard Col-0 ecotype and analyzed $F_2$ progeny using the criteria of hybridization and visible symptoms, as shown in Table 1. In all experiments the efficiency of infection was 42%. Taking this efficiency into account, the En-2 resistance trait, scored by either visible symptoms or by hybridization signals, segregated ~3:1 (resistance/susceptible) in the $F_2$ progeny. The segregation data are consistent with a model that virus resistance in En-2 is largely conditioned by a dominant trait at a single locus. However, it is possible that other modifiers in the background of the two ecotypes may also contribute to the virus resistance trait.

## DISCUSSION

The plant skeleton hybridization procedure permits one to follow macroscopically the long-distance movement of CaMV during systemic infection. The technique also allows one to determine if the absence of visible symptoms in a virus infection is due to lack of virus or to the inability of the plant to produce viral symptoms. By employing this technique, we have found that several important parameters influence the pattern of CaMV movement in systemically infected plants. First, CaMV is transported systemically through phloem where it moves with the flow of photoassimilates (Leisner et al., 1992). In general, photoassimilates travel from source leaves to sink leaves and from older to younger leaves. Therefore, systemic CaMV infections spread from source leaves to sink leaves. Second, sink–source relationships change during plant

development as does the pattern of virus movement. Newly emerging leaves are sinks for photoassimilates and maturing leaves transform from sink leaves to source leaves, that is, from net importers of photoassimilates to net exporters of photoassimilates. Any given leaf is transformed from an importer to an exporter in a developmental wave that moves basipetally down the leaf.

Third, like photoassimilates, CaMV does not invade leaves that have passed through the sink-to-source transition. However, in contrast to photoassimilates, CaMV is restricted from developing leaves at an earlier stage in development. In turnip, CaMV is no longer imported by developing leaves when the leaves reach 30% of their full length (Leisner et al., 1992). On the other hand, leaves stop importing photoassimilates when they reach 70% of their full length. During the time when a leaf is initiated until it reaches 30% of full length, the uptake of CaMV is lost in a basipetal fashion. Fourth, there is a latent period following the time when a leaf is inoculated until the time when the virus exits the leaf. Presumably during this period, the virus replicates and moves from cell to cell until it enters the phloem vasculature. By removing turnip leaves at various times after inoculation, we found that it takes 5 days for CaMV to exit from an inoculated leaf (Leisner et al., 1992). Using the plant skeleton hybridization technique in Arabidopsis, we could not detect the establishment of systemic infection outside the inoculated leaf until 14 to 18 days after inoculation. During this time, the region of the plant accessible to virus changes because the inoculated plant continues to produce new leaves while others mature. Therefore, the region of the plant that would have been accessible to a systemically transported virus at the time of inoculation is not the same region that is actually accessible to the inoculated virus.

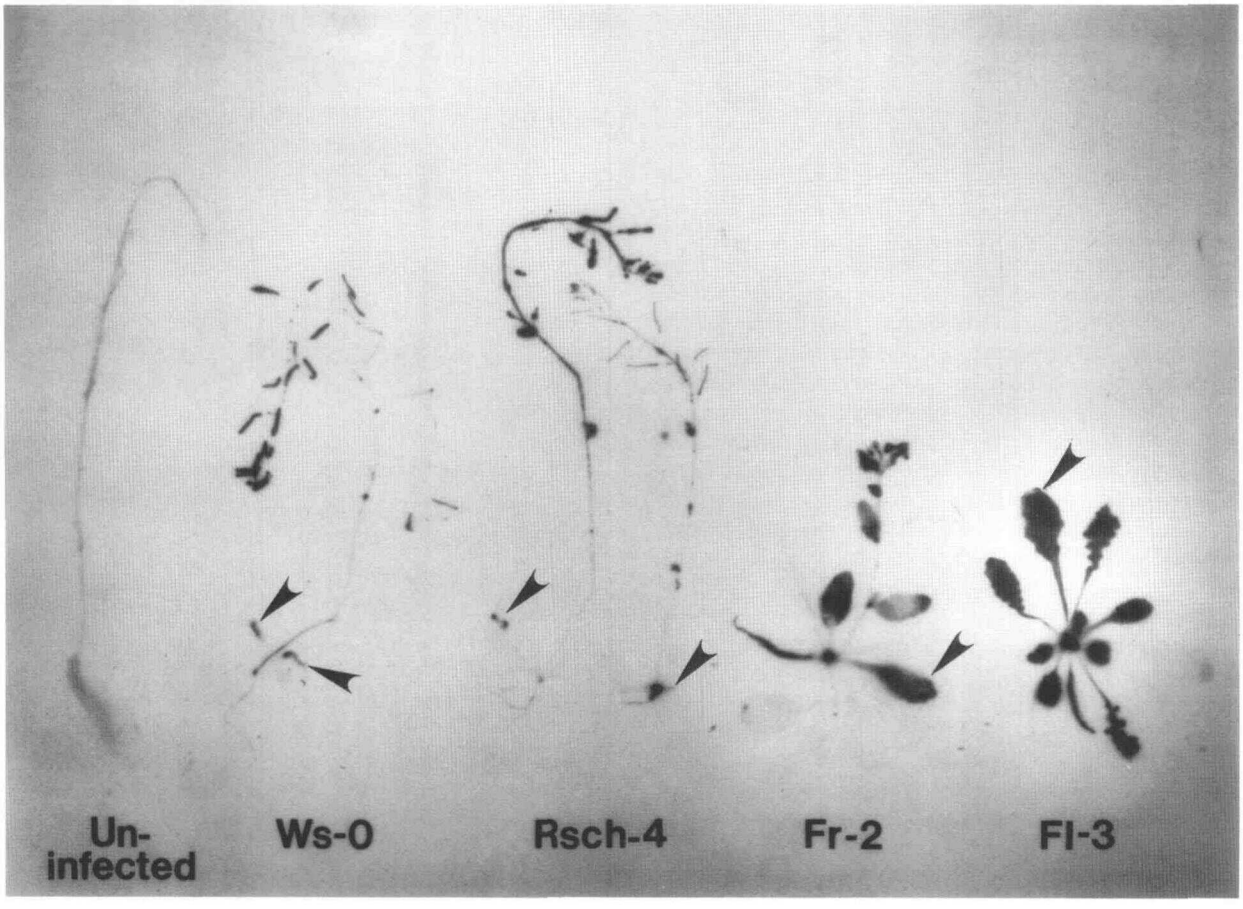


**Figure 6.** Distribution Pattern of CaMV DNA in Various Arabidopsis Ecotypes.

The ecotypes were inoculated 14 days after vernalization on leaves 2 to 4 with CaMV isolate CM4-184. Plants were harvested 35 days postinoculation and prepared for plant skeleton hybridization. The uninfected control is a plant of the Rsch-4 ecotype. Inoculated leaves remaining at the time of harvest are indicated with arrows. In the righthand plant of the pair of plants of the Rsch-4 ecotype, the inoculated leaf had mostly deteriorated by the time of harvest; however, the petiole of that leaf remains and is marked with an arrow.

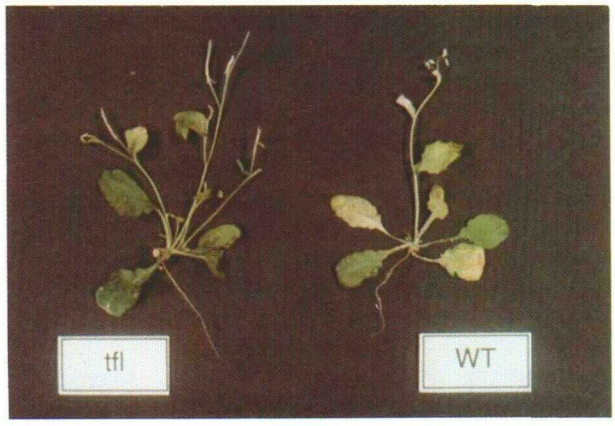


***Figure 7.*** *Pattern of Symptoms in an Early Flowering Arabidopsis* Mutant.

*tfl* mutant and wild-type (WT) Col-0 ecotype plants inoculated with CaMV isolate CM4-184 are shown. Plants were harvested 35 days postinoculation. The inoculated leaves senesced prior to harvest and are not shown.

The consequence of these effects is that during development, the region of the plant that is accessible to systemic infection from any given source leaf is progressively reduced. Under given growth conditions, many of the uninoculated rosette leaves in the standard ecotype, Col-0, are accessible to CaMV spreading from leaves 2, 3, and 4 inoculated at the four-leaf seedling stage. However, later in development, the rosette leaves are no longer sinks for photoassimilates and are inaccessible to virus. Thus, for the standard ecotype, the developmental window for systemic infection of rosette leaves is quite small. For rapidly developing or early flowering Arabidopsis ecotypes, the window for infection of rosette leaves is almost never open (if the plants are inoculated at the four-leaf stage). That is, by the time CaMV goes systemic in early flowering plants, all the rosette leaves may have matured beyond the point where they can import virus. Figure 9 shows this in a diagrammatic fashion. The earlier a plant flowers, the more localized to the upper part of the plant is the region accessible to the virus. Also, the region actually invaded by the virus is not the same as that accessible to the virus at the time of inoculation because a certain amount of time lapses until the virus exits the inoculated leaf.

Early flowering plants appear resistant to systemic virus infection, because the rosette leaves are not invaded by virus and do not show symptoms. The resistance results from a mismatch between the kinetics of virus movement and the rate of development of the infected plant. Although symptoms do not appear on uninoculated leaves in the early flowering plants, lesions appear as expected on the inoculated leaves. Therefore, in these plants there does not appear to be any impediment to virus replication of symptom production per se. Because this type of resistance is dependent upon the rate of plant development, we propose the term "developmental resistance" to describe it.

The implications are that any factor, genetic or environmental, that influences the rate of plant development would have ramifications on viral distribution and symptom severity. Others have reported differences in symptom severity at different times of the year with the same virus and host (Nono-Womdim et al., 1991). It is likely that differences in the light–dark cycle and temperature affect the rate of plant development at different times of the year. Our data predict that plants growing in the winter months (growing more slowly) would be more susceptible to the virus than those same plants grown during the summer months (growing more rapidly). Of course, this assumes that the rate of invasion of the phloem by the virus is the same at different times of the year. In at least one case this prediction has been borne out (Nono-Womdim et al., 1991). Because Arabidopsis plants have such a rapid life cycle, even small perturbations in environmental conditions could have dramatic effects on the apparent susceptibility to systemic CaMV infections.

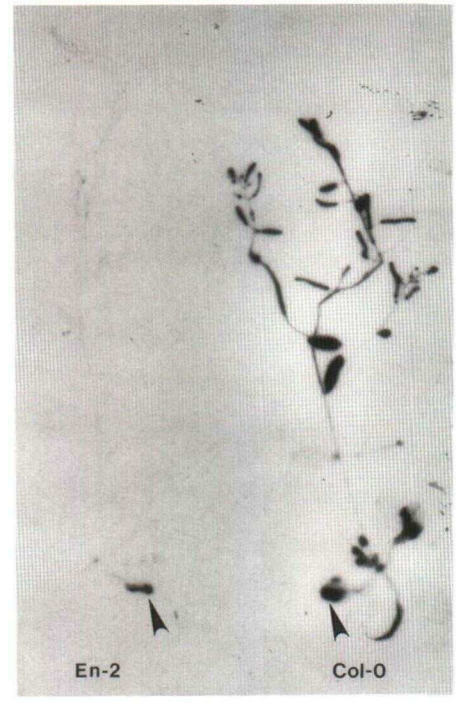


**Figure 8.** Long-Distance Movement of CaMV Is Blocked in Plants of the En-2 Ecotype.

The distribution pattern of viral DNA in susceptible and resistant Arabidopsis ecotypes inoculated with CaMV is shown. Arabidopsis plants were inoculated with CaMV isolate CM4-184 and were prepared for plant skeleton hybridization at 41 days postinoculation. Inoculated leaves are indicated by arrows. Left, Arabidopsis ecotype En-2, a resistant ecotype; right, Arabidopsis ecotype Col-0, a susceptible ecotype.

**Table 1.** Segregation of Virus Resistance in $F_2$ Progeny from Cross of Col-0 × En-2 Ecotypes

| | Raw Data | Corrected[a] | Expected[b] |
|---|---|---|---|
| Visible symptoms | 22[c] | 52 | 65 |
| No visible symptoms | 238 | 208 | 195 |
| Total | 260 | 260 | 260 |
| | | | |
| Hybridization signal[d] | 28 | 66 | 55 |
| No hybridization signal | 189 | 151 | 162 |
| Total | 217 | 217 | 217 |

[a] Correction based on the efficiency of infection (42%). Efficiency of infection determined for Arabidopsis Col-0 ecotype using plant skeleton hybridization.

[b] Expected numbers based on a 1:3 segregation ratio of susceptible-to-resistance traits. Visible symptoms $\chi^2 = 3.46$, $P = 0.07$; hybridization $\chi^2 = 2.94$, $P = 0.09$.

[c] Number of plants.

[d] Signal observed in $F_2$ progeny using plant skeleton hybridization technique.

It is important to understand that mature leaves which cannot be systemically infected by CaMV are still capable of supporting virus replication. Maule and coworkers have demonstrated that turnip leaves which have matured beyond the point where they can be systemically invaded can still be mechanically infected, that is, infected by direct inoculation (Maule et al., 1989). Furthermore, in leaves that were previously infected systemically, CaMV continues to replicate until the leaves reach full maturity. Thus, it appears that leaves which can no longer import virus are fully capable of supporting virus replication. A possible explanation for this phenomenon is that the infectious viral entity that moves through the phloem, whether it is a virus particle or nucleic acid–protein complex, is restricted from entering the vasculature in mature leaves. Because viruses move in the same vascular pathways as photoassimilates, viruses would be required to travel against the flow of photoassimilates to invade mature leaves. Another possibility is that the infectious entity might be able to invade the phloem vasculature of the mature leaf, but cannot exit and multiply in the mesophyll cells of the leaf.

Bearing in mind the effects of the rate of plant development on systemic CaMV symptoms, we screened Arabidopsis ecotypes for bona fide CaMV resistance. In one Arabidopsis ecotype, En-2, one CaMV isolate (CM4-184) does not produce a systemic infection, although it appears to replicate and move from cell to cell in inoculated leaves. Using the plant skeleton hybridization technique, we found virus in the petioles of inoculated leaves, but not in other leaves. The resistance character in Arabidopsis En-2 segregates as a single, dominant trait; therefore, in the future it may be possible to define the resistance trait in molecular terms. The resistance in En-2 does not appear to be a standard defense response, because we observed no evidence of necrosis in the inoculated leaf.

Inhibition of viral movement appears to be a common form of resistance, as illustrated by Beier and coworkers (Beier et al., 1977). They screened more than 1000 cultivars of cowpea and found 54 cultivars resistant to cowpea mosaic virus. Of those 54 lines, only one blocked virus replication. Pepper varieties resistant to cucumber mosaic virus allowed the replication and cell-to-cell spread of the virus in inoculated leaves, but limited viral spread to the upper parts of infected plants (Dufour et al., 1989). If the virus did exit the inoculated leaf, it was confined to one or two phloem bundles present within the petiole, stem, and root; however, it never invaded the parts

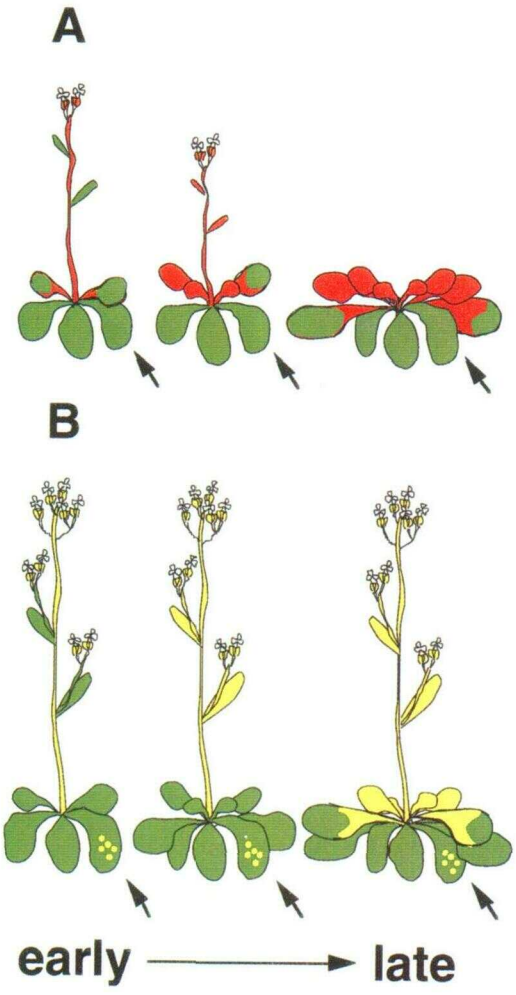


**Figure 9.** Effects of Plant Development (Time to Flowering) on CaMV Long-Distance Movement in Arabidopsis Plants.

**(A)** Representation of the region accessible to CaMV in immature plants that develop at different rates. Red represents region accessible to systemic CaMV invasion. Arrows indicate the inoculated leaf.

**(B)** Virus distribution in mature plants that developed at different rates. Yellow represents viral symptoms.

of the plant above the inoculated leaf. Also, certain partially resistant cultivars exhibited a delay in the rate of systemic symptom formation and probably long-distance movement. Certain maize varieties have also been shown to inhibit the long-distance spread of maize dwarf mosaic virus (Lei and Agrios, 1986; Law et al., 1989). In this case, the virus propagated well in the inoculated leaf but had difficulty exiting the inoculated leaf. Also, if it did exit the inoculated leaf, the virus was prevented from invading the parts of the plant above the inoculated leaf. In a soybean variety resistant to cowpea chlorotic mottle virus, the virus was found in all cell types of the inoculated leaf except for vascular tissues (Goodrick et al., 1991). In upper noninoculated leaves of the resistant variety, the virus was rarely found. This suggests, again, that the virus was prevented from invading the veins of the resistant variety. In at least one case, the resistance of a cowpea cultivar to cowpea chlorotic mottle virus was a single, dominant trait (Kuhn et al., 1981). The En-2 ecotype of Arabidopsis may be another example of this phenomenon.

In this paper we have shown two different types of resistance: one an apparent resistance due to a mismatch between the rate of plant development and the kinetics of virus movement, and another form of resistance due to an inhibition of plant virus long-distance movement. It is interesting that although the mode of "resistance" is different for these two types, both are manifestations of restricted long-distance movement.

## METHODS

### Ecotypes Used

The *Arabidopsis thaliana* ecotypes used in this study were obtained from Dr. Robert Last at the Boyce Thompson Institute for Plant Research and from the Arabidopsis Information Service, Frankfurt, Germany. The *tfl1-1* mutant was a gift from Dr. D. R. Meeks-Wagner, University of Oregon, Eugene. Arabidopsis seeds were planted in moistened Redi Earth mix (Fleco Quality Products, Seneca Castle, NY) and vernalized for 2 weeks at 4°C in 4-inch-diameter fiber pots covered with plastic wrap. The plants were then placed in a light room under continuous illumination at 60 $\mu$E $m^{-2}$ $sec^{-1}$ of PAR at 21°C. Three days after the seeds germinated, the plastic wrap was removed. Plants were subirrigated by soaking pots in a plastic flat filled with water for 3 to 4 hr. After 14 days, the plants developed four to six true rosette leaves and were thinned to 20 plants per pot. Plants were then inoculated with virus on the day after thinning.

### Viral Isolates, Inoculation, and Growth Conditions

Cauliflower mosaic virus (CaMV) isolate CM4-184 was maintained by serial passage in *Brassica campestris* var rapa cv Just Right (turnips) in a greenhouse. Twenty Arabidopsis plants per pot were mechanically inoculated with cell sap prepared by grinding infected turnip leaves in 10 mM potassium acetate buffer, pH 7.2, at 3 mL of buffer $g^{-1}$ tissue. Celite was added at 6 mg $mL^{-1}$ of cell sap, and 5 $\mu$L of the cell sap was rubbed with a plastic spatula on three leaves per plant (leaves 2, 3, and 4) unless otherwise indicated. Leaves were rinsed with water 5 min after inoculation, and inoculated plants were incubated for 8 hr at room temperature under room lights. Plants were then grown in a growth chamber under a 12-hr light and 12-hr dark cycle at 19°C. The total photosynthetic active radiation of ~50 PAR was provided by fluorescent and incandescent lamps. Plants were rotated to different locations within the chamber every 2 days to assure even lighting. Plants were observed every day for systemic symptoms.

### Plant Skeleton Hybridization

Arabidopsis plants were removed from soil and subjected to leaf skeleton hybridization as described by Melcher and colleagues (1981, 1989). Briefly, the plants were washed, put into plastic bags with 100 to 200 mL of ethanol, and allowed to sit overnight at room temperature. The next day, the ethanol was removed and 5 to 10 mL of a solution consisting of 0.1 mM sodium azide, 0.1% SDS, and 0.1 mg $mL^{-1}$ of proteinase K was carefully added to the bag containing Arabidopsis plants and incubated at 37°C overnight. The following day, the plants were rinsed four times with water, treated with 100 to 200 mL of 0.5 M NaOH, 1.0 M NaCl for 20 min, then with 100 to 200 mL of 1.0 M Tris-HCl, pH 7.5, 1.5 M NaCl for 20 min, rinsed in 2 × SSC (1 × SSC is 150 mM NaCl, 15 mM sodium citrate), air dried, and baked under vacuum for 2 hr at 80°C.

The plants were then hybridized in a solution consisting of 2 × SSC, 0.1% SDS, 1.0 mg $mL^{-1}$ of heat-denatured salmon sperm DNA, 1 × Denhardt's solution (1 × Denhardt's solution is 0.02% PVP, 0.02% BSA, and 0.02% Ficoll), and $10^4$ to $10^6$ dpm of random hexamer–labeled viral probe per milliliter of hybridization solution. The viral probe was the 8-kb viral DNA insert from the plasmid pLW414 (the cloned genome of the CM4-184 isolate of CaMV; Howell et al., 1980), which was labeled with an Amersham MultiPrime DNA labeling kit according to manufacturer's instructions.

Plants were washed two times with 50 mL of 2 × SSC and two times with 100 mL of 2 × SSC, all at 68°C. Plants were then mounted on a sheet of mylar plastic, wrapped in plastic wrap, and autoradiographed. Normal exposures ranged in time from 1 to 6 days.

### Photoassimilate Labeling

The pattern of photoassimilate translocation within Arabidopsis plants was determined by labeling the third true leaf with $^{14}CO_2$ (Turgeon, 1989). The leaf was enclosed within a polyethylene bag and incubated for 5 min with $^{14}CO_2$ generated by injecting excess 80% lactic acid onto $Na_2{}^{14}CO_3$ (6.6 MBq $mmol^{-1}$) in the bag. After translocation for 1 hr, the whole plant was placed into a glass Petri dish and frozen by covering with powdered dry ice. Throughout the experiment, the plant was illuminated by a water-filtered 1000-W metal halide lamp (M1000/C/U Metalarc; Sylvania, Danvers, MA) providing 400 $\mu$mol $m^{-2}$ $sec^{-1}$ PAR at the level of the labeled leaf. Frozen leaves were lyophilized (Virtis freeze dryer; Virtis Co., Gardiner, NY) for 7 days at −30°C (condenser at −60°C). Lyophilized plants were flattened between polished steel plates in a large vise, and the flattened plants were exposed for 7 days to x-ray film (Hyperfilm-βmax; Amersham International). Further details are given by Weisberg et al. (1988).

### Genetic Analysis

Arabidopsis ecotype Enkheim (En-2) plants were used to pollinate the glabrous mutant (*gl-1*) of the Columbia (Col-0) ecotype. The *gl-1*

mutant develops symptoms identical to wild-type Col-0 plants, and the *gl-1* mutation was used to check the fidelity of the cross. $F_1$ plants were grown under continuous light at 60 PAR at 20°C and selfed. Seeds were recovered and pooled to produce the $F_2$ seed population that was analyzed for the segregation of the En-2 resistance trait. The $F_2$ plants were inoculated as above with virus at the four-leaf stage on leaves 2, 3, and 4 with CaMV CM4-184. The plants were then grown under a 12-hr light and 12-hr dark cycle at 50 PAR and 19°C, and scored for the production of visible symptoms at 36 days postinoculation.

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