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Characterization of Cauliflower Mosaic Virus (CaMV) Resistance in Virus-Resistant Ecotypes of *Arabidopsis*

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Two *Arabidopsis* ecotypes are resistant to systemic infection by cauliflower mosaic virus (CaMV), a plant pararetrovirus. *Arabidopsis* ecotype Enkheim-2 (En-2) is highly resistant to CaMV infection while Bla-14 is more weakly resistant. CaMV resistance in En-2 can be largely attributed to the action of a single semidominant gene called *cauliflower mosaic virus resistance1* (*CAR1*), located at a locus on chromosome 1. Resistance in Bla-14 is tightly linked to *CAR1* and may be due to a weak allele at the same locus or another gene in a gene cluster. A quantitative polymerase chain reaction assay in conjunction with replication- and movement-incompetent viral mutants was used to determine whether virus replication or movement is affected in the resistant ecotypes. The pattern of accumulation of the wild-type virus in the resistant ecotype, En-2, was similar to that of a movement-incompetent CaMV mutant, suggesting that *CAR1* interferes with or fails to support CaMV movement. CaMV-inoculated En-2 plants do not show visible signs of a hypersensitive response. However, indicators of an induced defense response do appear in CaMV-infected En-2 plants, such as the activation of pathogenesis-related protein gene expression and the production of camalexin, an *Arabidopsis* phytoalexin. Defense responses induced chemically or by mutation in the susceptible ecotypes delayed and reduced the severity of a CaMV infection. These findings suggest that *CAR1* acts either in the susceptible ecotype to support virus movement or in the resistant ecotype to signal a defense response.

Additional keywords: movement protein, quantitative polymerase chain reaction, systemic acquired resistance (SAR) response.

Natural disease resistance in plants has been classified as follows: (i) nonhost resistance (or immunity), in which all individuals of a species are unaffected; (ii) cultivar resistance, in which some members of the species have a gene or genes that confer some degree of resistance; and (iii) induced or acquired resistance, in which the resistance is induced, but the induced state is not heritable (although the capability to express the induced resistance might be heritable) (Fraser 1990). In cultivar and/or induced resistance the pathogen replicates limitedly, while in nonhost resistance the pathogen is not thought to replicate. In addition, cultivar and/or induced resistance is commonly determined by a single dominant gene, while nonhost resistance is more likely to be multigenic (Dawson and Hilf 1992). Fraser (1990) proposed three general mechanisms of resistance: (i) active responses, in which the host mounts a defense to a pathogen; (ii) negative or passive response, in which the host lacks factors that are required for susceptibility; and (iii) physical or chemical barriers to initial infection. A classic example of active responses to viral infection is the hypersensitive response (HR) elicited by tobacco mosaic virus (TMV) in cultivars of *Nicotiana sylvestris* bearing the N′ gene (Dawson and Hilf 1992).

Active defense responses involve processes in which the pathogen is recognized and defense responses are deployed, such as an HR or a systemic acquired resistance (SAR) response (Staskawicz et al. 1995). Recently, host genes involved in active defense responses in plants have been cloned, e.g., the N gene in *Nicotiana tabacum* cv. Samsun NN (Whitham et al. 1994). The N gene product appears to be a component of the recognition and/or signaling pathway involved in the defense response to TMV infection.

Ecotype (cultivar) resistance to viruses has been identified in *Arabidopsis* (Lee et al. 1994). For example, resistance to beet curly top geminivirus (BCTV) has been identified in various ecotypes, such as Ms-0 and Pr-0. Studies of viral DNA replication in excised inflorescence pieces demonstrated that BCTV replicates in tissues from these resistant ecotypes, suggesting that resistance was due to a block in virus movement. BCTV does not produce necrotic symptoms in the resistant ecotypes; therefore, resistance does not appear to be based on the deployment of an HR.

Ecotype resistance to turnip crinkle virus (TCV) has also been described in *Arabidopsis*. Most *Arabidopsis* ecotypes are susceptible to TCV; however, resistance has been reported in ecotypes Di-0 (Simon et al. 1992) and Di-17 (Dempsey et al. 1993). Inoculation of Di-0 with TCV produces small necrotic lesions in some inoculated plants, but the virus spreads throughout the inoculated leaf and to the opposite leaf. Inoculation of Di-17 with TCV generates an HR in which necrotic lesions are produced, and host genes encoding pathogenesis-related (PR) proteins are induced. The resistance to TCV in Di-17 is more complete than in Di-0, and the HR is more intense.

Resistance to cauliflower mosaic virus (CaMV) was also found in a survey of various ecotypes of *Arabidopsis* (Leisner

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and Howell 1992). Much of the apparent resistance in different ecotypes was a "developmental resistance" in which the rapid growth of the plant outpaced the spread of the virus (Leisner et al. 1993). However, resistance to CaMV in three ecotypes was not simply due to developmental resistance. In one of the ecotypes, Enkheim-2 (En-2), virus was observed in inoculated leaves, but did not appear to move systemically. CaMV resistance in En-2 was shown to be a single Mendelian trait.

In this paper we describe the characteristics of CaMV resistance in two resistant ecotypes with particular focus on En-2. We report that virus resistance in ecotype En-2 is conditioned by a single semidominant gene called *cauliflower mosaic virus resistance1 (CAR1).*

RESULTS

CaMV resistance in *Arabidopsis* ecotypes.

Arabidopsis plants of the En-2 and Blanes-14 (Bla-14) ecotypes are resistant to systemic CaMV infection when sap-inoculated with CaMV (isolate CM1841) from infected turnips (Leisner and Howell 1992; S. M. Leisner, unpublished observations). En-2 is also resistant to CaMV delivered by agroinoculation; however, resistance to CaMV in Bla-14 breaks down following agroinoculation (not shown). The difference appears to relate to the effectiveness in delivery of the infectious agent by agroinoculation. The frequency of infection of plants by CaMV transmitted by sap from turnip to susceptible ecotypes (Col-0) of *Arabidopsis* was around 40% (Leisner et al. 1993). However, the agroinoculation vector (p1.5CaMV) developed for these studies was very potent (Fig. 1), and the frequency of infection in large populations of the susceptible Col-0 ecotype exceeded 99% (data not shown.)

In susceptible Col-0 plants, CaMV infection produced mosaic symptoms and frequent stunting of flower stalks (Fig. 2). Vein-clearing symptoms were usually only seen on the newer rosette leaves and the cauline leaves of the flower stalk. Older rosette leaves were not infected because there is a latent period following inoculation during which virus replicates and moves out of the inoculated leaves (Leisner et al. 1992). Following the latent period, only the newer rosette leaves and the flower stalks are nutrient sinks and are susceptible to systemic CaMV infection. Neither vein-clearing symptoms nor defense response indicators, such as necrotic lesions, were observed on the resistant En-2 plants (Fig. 2).

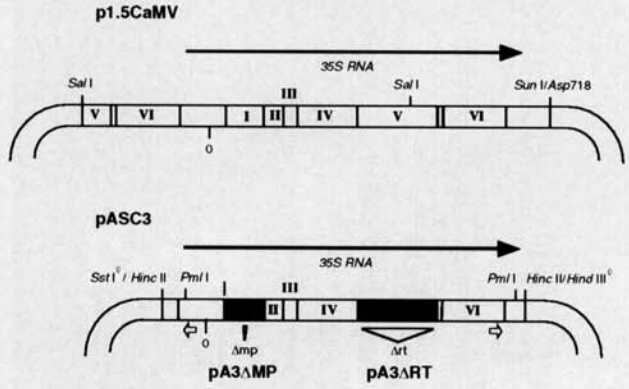

Fig. 1. Map of the agroinoculation vectors. Vectors contain partial multimers of the cauliflower mosaic virus (CaMV) (CM1841) genome (shaded segment) in the *Agrobacterium* binary vector Bin19 (nonshaded segment). Vector p1.5CaMV was employed for routine agroinoculation experiments and pASC3 was used for quantitative polymerase chain reaction (PCR) analysis. PCR primers (short unfilled arrows, not drawn to scale) detected the circularized form of the CaMV genome. A vector (pA3ΔMP) with an in-frame deletion (ΔMP) in the movement protein gene (gene I) was generated by deleting nucleotide position 741 to 906 in the CaMV genome (Gardner et al. 1981). A reverse transcriptase deletion mutant (ΔRT) was produced by creating a deletion in gene V at nucleotide position 3778 to 5517, resulting in mutant vector pA3ΔRT.

Genetic characteristics of CaMV resistance.

We reported in an earlier study that CaMV resistance in ecotype En-2 was a single, dominant trait as determined from the segregation of resistance in crosses with the susceptible ecotype (Col-0) (Leisner et al. 1993). In that study, seedlings were sap-inoculated with virus and scored for resistance by the appearance of symptoms. However, when F_1 hybrids in crosses of En-2 × Col-0 were agroinoculated with CaMV, the F_1 seedlings developed mild symptoms of virus infection. Thus, the CaMV resistance in ecotype En-2 appeared to be semidominant over susceptibility in Col-0, and F_1 hybrids were intermediate in resistance, i.e., showed mild symptoms. In F_2 progeny, resistance segregated about 1:2:1::susceptible:intermediate resistance:full resistance (data not shown). Scoring progeny as susceptible or showing intermediate resistance was confirmed in a number of cases by analyzing F_3 families derived from selfing individual F_3 progeny. In crosses with Landsberg erecta (Ler), CaMV resistance in En-2 also appeared to be semidominant. However, vein-clearing symptoms were very mild in F_1 hybrids, most likely because the Ler parent itself showed milder symptoms of CaMV infection than Col-0.

Virus resistance in En-2 was mapped by crossing the resistant ecotypes En-2 and Bla-14 with the susceptible ecotypes Col-0 and Ler. The mapping population was largely generated from susceptible F_2 progeny; however, all three phenotypes—susceptible, intermediate resistant, and fully resistant—were tested in most crosses.

CaMV resistance in En-2 was mapped with cleaved, amplified, polymorphic sequences (CAPS) and microsatellite markers (Bell and Ecker 1994; Konieczny and Ausubel 1993). It was found that nga111, a chromosome 1 microsatellite marker, was loosely linked to CaMV resistance in F_2 progeny of En-2 × Col-0, a cross in which the parents were polymorphic for nga111 (Table 1). Further mapping with the CAPS marker

Table 1. Mapping cauliflower mosaic virus (CaMV) resistance in *Arabidopsis* ecotypes En-2 and Bla-14

Marker[a]	F2 progeny scored (no.)	Recombinant fraction[b]
Cross 1: En-2 × Col-0		
nga111	108	25.1
Cross 2: En-2 × Ler		
GAP-B	57	10.7
nga128	752	3.7
Cross 3: Bla-14 × Col-0		
nga 128	62	3.2

[a] Microsatellite markers nga111 and nga128 described by Bell and Ecker (1994); cleaved, amplified, polymorphic sequence marker GAP-B described by Konieczny and Ausubel (1993).

[b] Recombinant fraction calculated with Mapmaker II (Lander et al. 1987).

GAP-B, the microsatellite marker nga128, and the F_2 progeny from cross 2 (En-2 × Ler), revealed that CaMV resistance was tightly linked to nga128 (χ = 3.7, Table 1). The virus resistance locus in En-2 was called *CAR1*.

CaMV resistance in Bla-14 was mapped in a similar way with a mapping population generated from cross 3 of Bla-14 × Col-0. Since the resistance trait in Bla-14 is much weaker than in En-2, resistance in Bla-14 was more difficult to score. However, the difference in resistance between Bla-14 and Col-0 was accentuated by agroinoculating at a later stage. It was found that the CaMV resistance trait in Bla-14 was also located on chromosome 1, linked to nga128 (Table 1). The resistance trait in Bla-14 showed comparable linkage to nga128 (χ = 3.2), as did the trait in En-2. Therefore, the CaMV resistance traits in the two ecotypes are tightly linked, or the trait in Bla-14 may be allelic to *CAR1*.

Virus replication in susceptible and resistant ecotypes.

To determine the extent to which virus replicates in the resistant ecotypes, a quantitative polymerase chain reaction (PCR) assay was developed to measure virus DNA accumulation. The PCR assay was set up to detect the appearance of virion DNA without interference from CaMV DNA in the agroinoculum. For this purpose, a new agroinoculation vector, pASC3, with shorter terminal repeats was developed for the assay (Fig. 1). This vector allowed for the use of PCR primers to generate a DNA fragment only when the viral genome was replicated and circularized on itself (A. Callaway and S. H. Howell, in preparation).

The PCR assay was used to measure CaMV accumulation in the susceptible ecotypes Col-0 and Ler and in the resistant ecotypes En-2 and Bla-14. The assay was highly discriminating in that no signal was observed in inoculated leaves sampled immediately after agroinoculation. (The limit of detection in the assay was ≈1 × 10^4 CaMV DNA molecules per mg of fresh weight tissue.) Four days post agroinoculation (DPI), detectable levels of viral DNA were observed in the inoculated leaves (rosette leaves) from all of the ecotypes (Fig. 3). At 8 DPI, viral DNA continued to accumulate in the rosette leaves, particularly in the susceptible Col-0 ecotype. Visible symptoms (vein clearing) were not detected at this stage of infection. By 14 DPI, virus accumulation peaked in rosette leaves of the Col-0 ecotype, reaching levels of 5 × 10^9 DNA molecules per mg of fresh weight. By this time, the plants had bolted and visible symptoms were beginning to appear, particularly in the systemically infected leaves (cauline leaves) in the Col-0 and Ler ecotypes. Viral DNA also appeared in cauline leaves, although the levels were not as high as in rosette leaves (when expressed on a per fresh weight basis). Viral DNA accumulated to somewhat lower levels in the rosette leaves of Ler, and peaked somewhat later (at 21 DPI). Likewise, the amount of viral DNA that accumulated systemically in cauline leaves was somewhat lower and more delayed than in Col-0.

In contrast, the accumulation of viral DNA was considerably reduced, but by no means eliminated in the resistant ecotypes. In rosette leaves, the weakly resistant Bla-14 ecotype accumulated about 30% and ecotype En-2 accumulated only 10% of the levels of viral DNA (5 × 10^8 DNA molecules per mg of fresh weight) compared with the Col-0 ecotype. Viral DNA also appeared in systemically infected cauline leaves of the En-2 ecotype although the levels of virus DNA were ≈7%

Fig. 2. Susceptible (Col-0) and resistant *Arabidopsis* ecotypes agroinoculated with p1.5CaMV at 21 days postinoculation. Inset shows detail of vein-clearing symptoms on Col-0 leaf and no symptoms on En-2 leaf.

the levels in cauline leaves of the Col-0 ecotype. Vein clearing symptoms were not detected in infected En-2 plants, but did appear in Bla-14 plants. Thus, the levels of virus accumulated in Bla-14 appear to exceed the threshold needed for the production of symptoms, whereas virus levels in En-2 remained below this threshold.

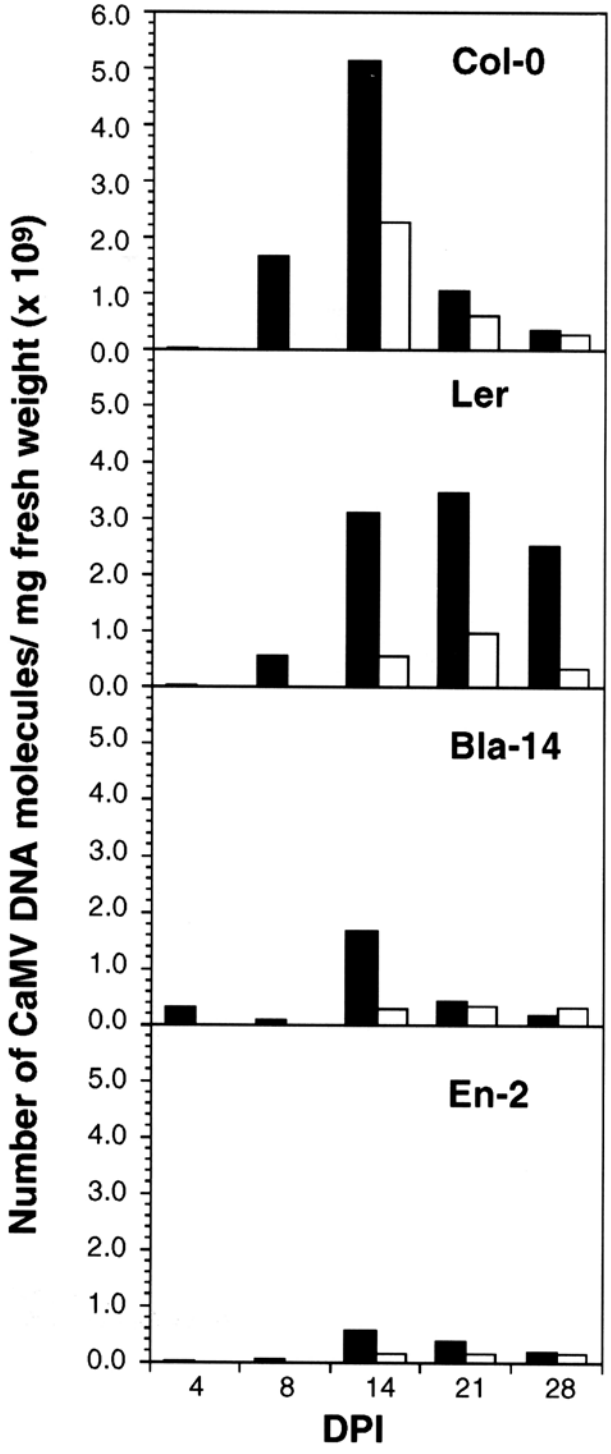

Fig. 3. Time course of cauliflower mosaic virus (CaMV) accumulation in various *Arabidopsis* ecotypes agroinoculated with wild-type CaMV (pASC3). A quantitative polymerase chain reastion (PCR) technique was used to measure the number of CaMV molecules per mg of fresh weight in extracts from inoculated rosette leaves (shaded bars) and systemically infected cauline leaves (open bars). Cauline leaves could only be sampled after flower stalks emerged (10 to 14 days postinoculation). Number of CaMV DNA molecules was determined by comparison to an internal standard in the PCR assay.

Accumulation of replication- and movement incompetent viruses.

To determine what steps in the virus infection process are defective in the resistant ecotypes, the accumulation of replication- and movement-incompetent mutant viruses was compared in the susceptible and resistant ecotypes. The movement-incompetent (ΔMP) and replication-incompetent (ΔRT) mutant viruses have deletions in genes I (movement protein gene) and V (reverse transcriptase gene), respectively. In the susceptible Col-0 ecotype, wild-type virus levels reached nearly 10^9 viral DNA molecules per mg of fresh weight of seedling at 8 DPI (Fig. 4). At 0 to 2 DPI the amount of virus DNA was below the limit of detection in this assay. At 3 DPI, the levels of wild-type virus DNA were similar in the Col-0 and En-2 ecotypes; however, from 4 to 8 DPI, virus levels rose more sharply in the susceptible ecotype than in the resistant ecotype. There was no detectable accumulation of ΔRT in either the susceptible or resistant ecotypes. Although wild-type virus replication was not as robust in En-2 as in Col-0, significant amounts of wild-type viral DNA accumulated in En-2 relative to the amount of ΔRT accumulated.

The accumulation of DNA from the ΔMP virus was compared in susceptible and resistant ecotypes (Fig. 4). In the susceptible Col-0 ecotype, it was found that ΔMP DNA leveled

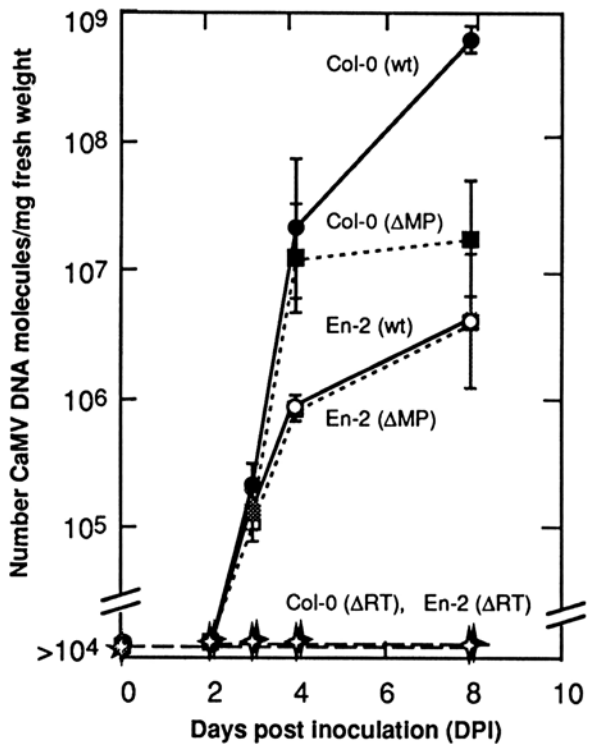

Fig. 4. Time course of accumulation of replication-incompetent (ΔRT) and movement-incompetent (ΔMP) viruses. Seedlings were agroinoculated with wild-type virus (pASC3), ΔRT (pA3ΔRT), or ΔMP (pA3ΔMP) mutant viruses. As in Figure 3, a quantitative polymerase chain reaction technique was used to measure the accumulation of virus in the susceptible ecotype Col-0 and the resistant ecotype En-2.

out at about 4 DPI. The accumulation of ΔMP DNA slowed at about the same time in the resistant ecotype, En-2. Interestingly, both wild-type and ΔMP viruses showed the same pattern of accumulation in En-2, suggesting that virus movement was either impaired or not supported in this ecotype.

Defense responses.

CaMV resistance in the resistant ecotypes could be due to defense responses or to passive resistance. To determine whether activation of a defense response could protect *Arabidopsis* from CaMV infection, seedlings were sprayed with a chemical inducer of SAR, 2,6-dichloroisonicotinic acid (INA)

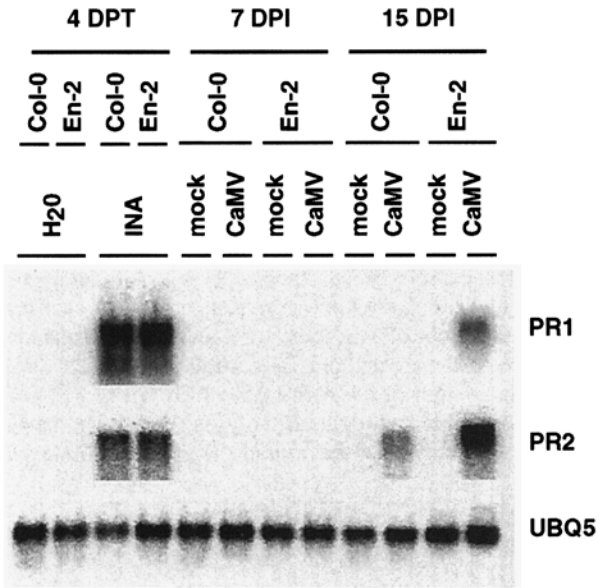

Fig. 5. Defense response gene expression in susceptible (Col-0) and resistant (En-2) ecotypes agroinoculated with cauliflower mosaic virus (CaMV) (p1.5CaMV). RNA was extracted from seedlings at various days postinoculation (DPI) and subjected to electrophoresis (5 μg of RNA per lane). Blots of the electrophoresis gels were hybridized to labeled probes of pathogenesis-related (PR) protein genes (PR1 and PR2) and ubiquitin 5 (UBQ5) used as a loading control. Control lanes for PR gene expression were generated by spraying seedlings with 0.25 mg of 2,6-dichloroisonicotinic acid (INA) or H_20 per ml and by extracting RNA 4 days post-treatment (DPT). Mock-inoculated plants were agroinoculated with a binary vector (Bin19) lacking a virus DNA insert.

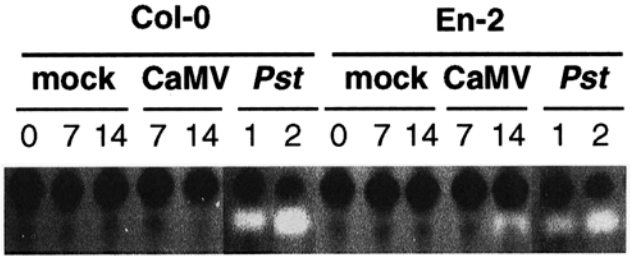

Fig. 6. Accumulation of camalexin, an *Arabidopsis* phytoalexin, in susceptible (Col-0) and resistant (En-2) ecotypes agroinoculated with cauliflower mosaic virus (CaMV). Extracts from inoculated *Arabidopsis* seedlings at various (0 to 14) days postinoculation were analyzed by thin-layer chromatography and camalexin visualized by UV illumination. Positive controls were produced by inoculating seedlings with *Pseudomonas syringae* pv. *tomato* DC3000/avrb1 (*Pst*). Mock-inoculated plants were agroinoculated with a binary vector (Bin19) lacking a CaMV DNA insert.

(Uknes et al. 1992). The appearance of symptoms in the susceptible Col-0 *Arabidopsis* ecotypes was delayed by about 2 days, and the severity of symptoms was reduced when seedlings were sprayed with INA at the time of or 4 days prior to agroinoculation with wild-type virus (Table 2). However, the conditions for protecting *Arabidopsis* from CaMV infection by INA application were not systematically optimized. More protection from CaMV infection was afforded by the mutation *cpr1* (*constitutive expressor of PR genes1*) (Bowling et al. 1994). In a susceptible ecotype background, *cpr1* delayed CaMV infection by 10 days and reduced symptom severity. Thus, either chemical or mutational induction of SAR will protect seedlings from CaMV infection. In mutants and transgenic plants with a compromised defense response, such as *npr1* (*nonexpressor of PR genes*) (Cao et al. 1994), symptoms appeared at the same time and with the same severity as the nonmutant Col-0 ecotype (Table 2). Likewise, the timing and severity of symptoms were similar in *nahG* transgenic plants compared with the parental Ler ecotype. *nahG*, a bacterial salicylate hydroxylase gene, prevents the accumulation of salicylic acid and the expression of SAR-like responses (Friedrich et al. 1995).

We determined whether CaMV infection in the resistant ecotype En-2 induced a defense response even though necrotic lesions, indicative of an HR, were not produced in resistant plants (Fig. 2). Other indicators of a defense response were monitored, such as activation of expression of PR gene expression. It was found that PR1 and PR2 were activated 4 days after treatment with the chemical inducer INA in both susceptible Col-0 and resistant En-2 ecotypes (Fig. 5). Therefore, En-2 plants appear as capable as Col-0 plants in activating defense genes in response to chemical inducers. In the resistant En-2 ecotype, PR1 and PR2 gene expression was activated at 15 DPI in response to CaMV infection (and not in response to mock agroinoculation). At this time, PR2 was expressed at higher levels than PR1 in En-2. In the susceptible Col-0 ecotype, PR2 expression was also activated at 15 DPI, but at lower levels than in En-2. PR1 expression was not activated at 15 DPI in Col-0, at a time when symptoms appeared in this ecotype.

The production of an *Arabidopsis* phytoalexin, camalexin, was also monitored to determine whether CaMV inoculation induces a defense response in resistant plants (Fig. 6). It was

Table 2. Effect of systemic acquired resistance–related responses on cauliflower mosaic virus (CaMV) infection in *Arabidopsis*

Genotype	Treatment	Delay in symptom appearance	Symptom severity[a]
wt (Col-0)	None	0 days[b] (n = 3)[c]	+++
wt (Col-0)	INA, –4 days[d]	2 days (n = 1)	+
wt (Col-0)	INA, 0 days	2 days (n = 1)	++
wt (Col-0)	INA, +4 days	0 days (n = 1)	+++
cpr1 (Col-0)	None	10 days (n = 3)	+
npr1 (Col-0)	None	0 days (n = 2)	+++
wt (Ler)	None	1 day (n = 3)	++
nahG (Ler)	None	1 day (n = 4)	++

[a] Symptom severity judged by the extent of vein-clearing symptoms in the population.
[b] Symtoms appeared at 11 days post inoculation.
[c] Number of pots analyzed. Each pot contains ≈100 plants.
[d] Seedlings were sprayed with 0.25 mg of 2,6-dichloroisonicotinic acid (INA) per ml at indicated days before or after agroinoculation.

found that the pathogen *Pseudomonas syringae* DC3000/avrB1 rapidly induced camalexin accumulation in both Col-0 and En-2. Camalexin was easily detectable in extracts at 1 DPI. In CaMV-inoculated seedlings, camalexin accumulated only in the En-2 ecotype, but was not detected until 14 DPI. In the susceptible Col-0 ecotype, camalexin accumulation was not detected even at longer times after CaMV inoculation and the appearance of symptoms (data not shown).

Leaves on agroinoculated seedlings were also inspected microscopically for the appearance of local defense responses, such as the accumulation of UV-fluorescent compounds (phenolics, etc.) In inoculated leaves on En-2 seedlings, but not in Col-0 seedlings, autofluorescent patchses began to appear at 7 DPI (Fig. 7). Within these patches, small spots and minor veins were UV-fluorescent. Similar patterns of callose accumulation were also observed only on the leaves of inoculated En-2 seedlings (data not shown).

DISCUSSION

Arabidopsis ecotypes En-2 and Bla-14 show differing degrees of resistance to CaMV (isolate CM1841). Neither ecotype produces symptoms when sap-inoculated with CaMV. However, ecotype En-2 is more resistant than Bla-14 to CaMV infection because resistance in Bla-14 can be broken by agroinoculation. In the resistant ecotypes, virus levels are reduced, but not eliminated. The levels of CaMV in infected En-2 are apparently below some threshold needed to produce symptoms. CaMV resistance in En-2 can largely be attributed to the action of the *CAR1* gene. The weaker CaMV resistance in Bla-14 may be due to the action of an allele of *CAR1* or to a closely linked gene. No other PR gene has been reported to map in this region of the *Arabidopsis* genome. Thus, it is likely that *CAR1* represents a new resistance gene.

Resistance in En-2 appears to affect virus movement and the development of a systemic infection. Virus (DNA) accumulates rapidly during the first few days of infection in the resistant ecotype, before the infection goes systemic. The accumulation of ΔMP virus in En-2 was similar to that of wild-type virus, suggesting that the systemic movement of wild-type virus is impaired in En-2. These observations are largely consistent with our previous findings regarding whole-mount in situ hybridization, which showed that virus did not move out of an inoculated leaf (Leisner et al. 1993). However, because we used a much more sensitive PCR technique in this study, it is clear that CaMV does move systemically in En-2 (into cauline leaves), although the virus levels are very low. Furthermore, we do not know if virus movement per se is restricted or if virus replication is blocked at about the time that the infection goes systemic.

CaMV infection of resistant ecotype En-2 induces an SAR-related defense response, activating the expression of defense response genes, such as PR1 and PR2 (Uknes et al. 1993a), and stimulating the accumulation of camalexin, a phytoalexin in *Arabidopsis* (Ausubel et al. 1995). The appearance of defense response indicators was quite surprising given that the virus does not produce necrotic lesions or an HR in the resistant ecotype. Usually SAR is produced as a consequence of a local HR (Uknes et al. 1992). However, there are other reports of non-necrotizing viruses generating SAR. For example, primary infection with TMV has been reported to protect cucumber from secondary challenges with the same virus or with TNV (Roberts 1982). However, more typical virus resistance involves necrotizing viruses, such as cucumber mosaic virus (CMV) strain Y in *Arabidopsis* ecotype C24. CMV strain 0, CMV(0), systemically infects ecotype C24, but CMV(Y) causes only a local infection and produces necrotic lesions that apparently limit the spread of the virus (Takahashi et al. 1994).

SAR-related defense responses induced by other means can provide resistance to CaMV in susceptible ecotypes. Spraying seedlings with INA at or before the time of agroinoculation delays the appearance and reduces the severity of CaMV symptoms. INA has been shown to be effective in protecting *Arabidopsis* from a virulent RNA virus, TCV (Uknes et al. 1993b). Even greater resistance to CaMV infection was seen in the constitutive defense response mutant, *cpr1* (Bowling et al. 1994). Therefore, if an SAR-like plant defense response is activated by CaMV, as it is in the resistant En-2 ecotype, then it might be effective in attenuating the virus infection. However, CaMV resistance afforded by INA or *cpr1* is either quantitatively or qualitatively different from the resistance in ecotype En-2 in that INA and *cpr1* only delay and reduce the severity of symptoms. The resistance in En-2 is more effective, since we have never observed a breakthrough in CaMV infection in En-2 even though virus accumulates to significant levels in the first few days after inoculation. However, CaMV infections do break through in Bla-14.

Several features about the resistance in En-2 are consistent with a "cultivar"-specific type of induced resistance associated with a defense response (Fraser 1990). First, CaMV resistance is ecotype-specific and monogenic. Nonhost resistance is usually species-specific and not necessarily monogenic. Second, CaMV resistance is semidominant, and an induced resistance trait is usually dominant (or semidominant) because the mechanisms are active, involving the induction of a defense response. Passive resistance traits are thought to be recessive because they are characterized by the absence of susceptibility factor(s). Third, CaMV does replicate locally in infected leaves, which is characteristic of an induced response

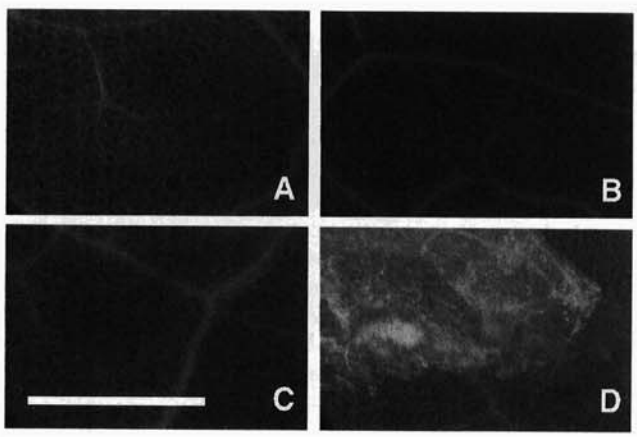

Fig. 7. Appearance of fluorescent compounds in leaves from agroinoculated *Arabidopsis* seedlings 7 days postinoculation (DPI). Photograph shows autofluorescence from UV illumination. Susceptible Col-0 ecotype **(A, B)**, resistant En-2 ecotype **(C, D)**. Mock agroinoculation with Bin-19 **(A, C)**. Agroinoculation with cauliflower mosaic virus (CaMV), p1.5CaMV **(B, D)**. Bar = 500 μm.

(Fraser 1985).

Although our results are consistent with the idea that *CAR1* conditions a defense mechanism, it is still possible that *CAR1* is a passive susceptibility trait. *CAR1* could confer susceptibility in the non-resistant ecotypes by providing the virus a host factor required for virus movement. The matter can be resolved in two ways. First, loss-of-function mutants could be generated in one ecotype or the other. It would be expected that in whichever ecotype the response to CaMV is a gain-of-function character, the trait could be disabled by knock-out mutation. Second, it could be determined whether CaMV resistance is a gain-of-function character by genetic complementation experiments. We are in the process of map-based cloning *CAR1*, and complementation tests should resolve the issue.

If *CAR1* allows for the rapid deployment of a defense response to CaMV infection, then the defense response may block virus movement. In that case, it will be very interesting to find out how the defense response blocks virus movement. However, it is possible that the defense response is the result and not the cause of a blockage in virus movement. The defense response appears to be deployed late during virus infection, after the infection goes systemic. We have shown that, if a defense response is chemically induced 4 days after agroinoculation, the response is ineffective in blocking systemic infection.

The use of a quantitative PCR technique to monitor CaMV infection is a significant advance (A. Callaway and S. H. Howell, in preparation). In this study, the PCR technique expanded the range of virus detectability over 5 orders of magnitude, allowing us to monitor the infection at very early stages. However, the present assay detects only viral DNA and does not discriminate between forms, i.e., whether the DNA is in the nucleus, in virions, in replicative intermediates, etc. It is possible that different CaMV DNA forms predominate in different hosts or under different conditions. Covey and Turner (1993) reported that different CaMV DNA forms accumulate at various stages of infection and different tissues. Adaptations of the technique are being developed to discriminate between the various forms.

MATERIALS AND METHODS

Arabidopsis ecotypes and virus inoculation conditions.

Arabidopsis thaliana ecotypes Columbia (Col-0), Landsberg erecta (Ler), Enkheim-2 (En-2), and Blanes-14 (Bla-14) were sown on pre-wet soil mix. Plants tested for virus resistance were grown at 21°C in a growth chamber under constant light at 50 $\mu E\ s^{-1}\ m^{-2}$. Two-week-old *Arabidopsis* plants were inoculated with CaMV, using sap from virus-infected turnips (*Brassica campestris* L. var. *rapa* cv. Just Right) or were agroinoculated with an *Agrobacterium tumefaciens* strain bearing an infectious multimeric copy of the CaMV 1841 genome (Grimsley et al. 1986).

For infection trials, a CaMV-genome-bearing vector plasmid (p1.5 CaMV) was constructed by ligating the 3.4-kb *SalI*/*Sun*I fragment from pCaMV10 (Gardner et al. 1981) containing the 3′-terminal half of gene V, all of gene VI, and the 5′ portion of VII, into the binary vector plasmid, pBIN19 (Bevan 1984), cut with *SalI*/*Asp*718 (Boehringer Mannheim, Indianapolis, IN) to create pCaMVCV-VII. pCaMVCV-VII was digested with *SalI* and ligated to the *SalI* fragment from pCaMV10 containing the entire CaMV 1841 genome to create p1.5 CaMV, an approximate 1.5-mer of the CaMV genome (Fig. 1).

The vector used in quantitative PCR experiments, pASC3, was constructed in two steps. The *Hinc*II fragment corresponding to positions 7016 to 7801 of the CaMV 1841 genome from pCaMV10 (Gardner et al. 1981) was ligated into *Hin*dIII /*Sst*I digested pBIN19 (Bevan 1984) in which the overhanging *Hin*dIII ends had been filled and the *Sst*I ends blunted with the Klenow fragment of DNA polymerase I (Promega, Madison, WI) to make pBIN35. The inserted CaMV fragment bears a *PmlI* site that is unique in pBIN35. A complete CaMV 1841 genome, excised from p1.5CaMV by *PmlI* digestion, was inserted into the *PmlI* site of pBIN35 vector to produce pASC3.

A movement-incompetent virus vector, pA3ΔMP, was constructed by digesting pCaMV10 with *Spe*I (NEB, Beverly, MA), filling in the overhanging ends with the Klenow polymerase (Promega, Madison, WI), heat-inactivating the polymerase, and then digesting with *Bam*HI (Promega). This fragment was ligated into *Eco*RI-digested pUCBR (a derivative of pUC19), the ends filled in with Klenow polymerase, (followed by heat-inactivation), then digested with *Bam*HI before ligation. The resultant pUCBRSpam was cut with *Nco*I, and the ends were filled with Klenow polymerase before digesting with *Bst*1107 I (NEB/Fermentas) and recircularizing with T4 DNA ligase (Promega) to generate pUCBR-SpamΔI. These manipulations result in an in-frame deletion (from nucleotide position 741 to 906 on the CaMV CM1841 map) (Gardner et al. 1981) of 45 amino acids in the central region of the movement protein that has been shown to be essential for activity (Thomas et al. 1993; Thomas and Maule 1995). This construct was confirmed by sequencing before transferring the deletion construct into pASC3 by replacing the *Bsi*WI (*Sun*I isoschizomer)/*Xho*I fragment with the *Bsi*WI/*Xho*I fragment from pUCBRSpamΔI to generate pA3ΔMP.

A replication-incompetent virus vector, pA3ΔRT, was constructed by digesting pASC3 with *SalI* (Promega) and incubating with Klenow polymerase to fill in ends, followed by digesting with *Hpa*I (NEB) and recircularizing with T4 DNA ligase, to produce pA3ΔCRT. In a screen of transformants, one clone was found in which additional sequences upstream of the *SalI* site were fortuitously deleted. Sequencing revealed that the deletion spanned the CaMV 1841 positions of 3778 to 5517. This removed most of the reverse transcriptase gene (V), leaving an open reading frame of only 50 amino acids before a stop codon. This clone was called pA3ΔRT.

The binary plasmids were introduced by electroporation into the *recA*$^-$ mutant of *A. tumefaciens* strain C58 (UIA143) (Farrand et al. 1989) cured of its native tumor-inducing (Ti) plasmid. A Ti-plasmid, pMP90, disarmed by insertion of a gentamicin marker (Koncz and Schell 1986) was introduced by electroporation into UIA143 to create the *Agrobacterium* strain UIA143/pMP90. This strain was transformed with p1.5CaMV and the resulting transformant UIA143/pMP90/p1.5CaMV called 1.5CaMV was used for most infectivity assays. Transformation of strain UIA143/pMP90 with pASC3 resulted in transformant UIA143/pMP90/pASC3 called ASC3, which was used in the quantitative PCR assays.

To agroinoculate *Arabidopsis* plants, a 36-h culture of one

of the *A. tumefaciens* strains was mixed with the abrasive, Celite (10 mg ml^{-1}). The suspension was sprayed onto seedlings at 2 weeks after germination (four-true-leaf stage) at 30 lb/in^2 with a homemade sprayer. Seedlings were rinsed with deionized water 15 min after inoculation and incubated overnight at room temperature and humidity conditions. Then the seedlings were returned to a growth chamber under a 16-h light/8-h dark cycle at 21°C. *A. tumefaciens* strain UIA143/pMP90 harboring the pBIN19 vector (without the CaMV genome insert) was used for mock inoculation.

Mapping of virus resistance.

Virus resistance traits in En-2 and Bla-14 ecotypes were mapped by crossing these ecotypes with the susceptible ecotypes, Col-0 or Ler. Mapping populations were generated by selfing F_1 progeny and testing F_2 for virus resistance. F_2 phenotypes were confirmed by segregation analysis in F_3 families. The chromosomal location of *CAR1* was determined by measuring the recombination frequency between the locus and microsatellites (Bell and Ecker 1994) that mark different *Arabidopsis* chromosomes. After the *CAR1* gene was located on a specific chromosome, CAPS (Konieczny and Ausubel 1993) and microsatellite markers were used for further mapping on that chromosome.

Virus detection assay.

Quantitative PCR and analysis of resulting products were carried out as described (A. Callaway and S. H. Howell, in preparation) and based on a procedure by Wang et al. (1989). In general, the method compares the yield of PCR product in a sample to an internal standard through a dilution series. Briefly, random leaves were collected from seedlings subjected to a specific inoculation procedure. Tissue was quick frozen in liquid nitrogen and crushed to a powder, and aliquots of the powder were immediately transferred to tared 1.5-ml microcentrifuge tubes. The tubes were weighed to determine tissue fresh weight. The tissue was ground in DNA extraction buffer with a power drill and plastic pestle. The ground samples were incubated at 55°C for 30 min before diluting 10× with sterile water and boiling for 8 min. The samples were stored at –20°C. One microliter of each sample was used as template for the PCR reactions. The primers for quantifying viral DNA and PCR conditions were designed so that viral DNA would not be amplified from the plasmid DNA in the inoculum but only from free-replicating virus. PCR products were resolved on 1% agarose gels. Detection was by post-staining with ethidium bromide or SYBR Green I (Molecular Probes, Eugene, OR) followed by image capture with the Eagle-Eye system (Stratagene, La Jolla, CA). Band intensities were measured with ImageCalc software (van de Lest et al. 1995). Quantities of virus were determined based on ratios of band intensities from the internal standard and the virus.

Plant defense response indicators.

To determine whether seedlings of the En-2 ecotype mount a defense response to CaMV infection, seedlings were agro-inoculated with CaMV as described above or sprayed with 0.25 mg of INA per ml and harvested by freezing in liquid N_2 at various times thereafter. RNA was extracted by grinding 100 mg of tissue in 200 µl of TRIzol reagent (GibcoBRL, Gaithersburg, MD) with a drill-mounted pellet pestle (Kontes, Vineland, NJ) in a 1.5-ml Eppendorf tube. After the tissue had been ground (≈30 s), 800 µl of TRIzol buffer was added, and RNA was extracted according to the manufacturer's instructions. RNA was subjected to electrophoresis on 1.2% agarose formaldehyde gels (Sambrook et al. 1989) and transferred to Hybond N^+ membranes (Amersham, Arlington Heights, IL). RNA was cross-linked to filters by UV irradiation (Stratagene Stratalinker) and subjected to hybridization with various probes in hybridization conditions modified from Church and Gilbert (1984). Filters were prehybridized in buffer containing 7% sodium dodecyl sulfate (SDS), 1% bovine serum albumin (BSA), 1 mM EDTA, and 250 mM sodium phosphate buffer (pH 7.2) and hybridized in the same solution at 65°C to 6×10^6 CPM of labeled probe per filter. Filters were washed twice for 10 min each at room temperature with 5% SDS, 40 mM sodium phosphate buffer (pH 7.2), and 2× for 15 min at 65°C with 1% SDS, 40 mM sodium phosphate buffer (pH 7.2).

The accumulation of the *Arabidopsis* phytoalexin camalexin was determined by a modification of two published protocols (Glazebrook and Ausubel 1994; Tsuji et al. 1992). Seven hundred microliters of 80% methanol was added to 70 mg of frozen tissue in a microcentrifuge tube. The samples were heated at 80°C for 15 min, and centrifuged at room temperature for 5 min. Five hundred microliters of the supernatant fluid was transferred to a new Eppendorf tube, and the methanol evaporated under vacuum. Each sample was adjusted to 100 µl with dH_2O and extracted twice with 100 µl of chloroform, and the pooled organic phase evaporated in the hood. The dried pellet was resuspended in 15 µl of chloroform, loaded onto a silica thin-layer chromatography (TLC) plate (VWR Scientific, South Plainfield, NJ) and developed with chloroform/methanol at 9:1. Camalexin was visualized under a long-wavelength UV lamp and photographed with Kodak Etkar 1000 film and a Kodak type 47 filter. The silica gel containing the camalexin was extracted twice with 1 ml of methanol and the camalexin quantified in a Perkin-Elmer (Foster City, CA) MPF-44B spectrofluorimeter (excitation at 315 nm, emission at 385 nm). Camalexin concentration was calculated by comparison with a standard curve obtained by using purified camalexin (provided by Jane Glazebrook).

UV-fluorescent compounds in inoculated leaves were localized by fluorescence microscopy with UV excitation according to Dietrich et al. (1994). Three leaves from plants subjected to a single treatment were fixed, prepared as whole mounts, and examined by microscopy.

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