Components of *Arabidopsis* Defense- and Ethylene-Signaling Pathways Regulate Susceptibility to *Cauliflower mosaic virus* by Restricting Long-Distance Movement

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We analyzed the susceptibility of *Arabidopsis* mutants with defects in salicylic acid (SA) and jasmonic acid (JA)/ethylene (ET) signaling to infection by *Cauliflower mosaic virus* (CaMV). Mutants *cpr1-1* and *cpr5-2*, in which SA-dependent defense signaling is activated constitutively, were substantially more resistant than the wild type to systemic infection, implicating SA signaling in defense against CaMV. However, SA-deficient *NahG*, *sid2-2*, *eds5-1*, and *pad4-1* did not show enhanced susceptibility. A *cpr5 eds5* double mutant also was resistant, suggesting that resistance in *cpr5* may function partially independently of SA. Treatment of *cpr5* and *cpr5 eds5*, but not *cpr1*, with salicyl-hydroxamic acid, an inhibitor of alternative oxidase, partially restored susceptibility to wild-type levels. Mutants *etr1-1*, *etr1-3*, and *ein2-1*, and two mutants with lesions in ET/JA-mediated defense, *eds4* and *eds8*, also showed reduced virus susceptibility, demonstrating that ET-dependent responses also play a role in susceptibility. We used a green fluorescent protein (GFP)-expressing CaMV recombinant to monitor virus movement. In mutants with reduced susceptibility, *cpr1-1*, *cpr5-2*, and *etr1-1*, CaMV-GFP formed local lesions similar to the wild type, but systemic spread was almost completely absent in *cpr1* and *cpr5* and was substantially reduced in *etr1-1*. Thus, mutations with enhanced systemic acquired resistance or compromised ET signaling show diminished long-distance virus movement.

Additional keywords: defense mutants, reactive oxygen, salicylic acid.

Considerable progress has been made in elucidating the host signaling and defense responses that are enacted when plants are infected by fungal and bacterial pathogens (Feys and Parker 2000; Hunt and Ryals 1996; Nimchuk et al. 2003). The central role of salicylic acid (SA) in mediating defense against a wide variety of biotrophic pathogens is now well established (Alvarez 2000). Increasing evidence indicates that these re-

sponses also are important in defense during both incompatible and compatible virus infections and that they appear to function through an *NPR1*-independent pathway (Kachroo et al. 2000; Murphy et al. 1999, 2001; Takahashi et al. 2002). These responses also can restrict the spread of some RNA viruses during compatible infections. Applications of SA, cyanide, and Antimycin A (AA) induced partial resistance to *Tobacco mosaic virus* (TMV) in susceptible tobacco cultivars and to *Turnip vein-clearing tobamovirus* (TVCV) in *Arabidopsis* (Chivasa et al. 1997; Wong et al. 2002).

On the basis of these and other experiments, Carr and co-workers postulated a virus-specific defense pathway in which reduced electron flow through the mitochondrion in response to increased SA levels stimulates the generation of reactive oxygen species (ROS). These act as signaling intermediates stimulating defense responses (Gilliland et al. 2003; Murphy et al. 1999; Singh et al. 2004). However, chemical inhibitors may exert pleiotropic effects when applied exogenously, and the ability to induce resistance chemically does not necessarily prove that such responses play an active role in limiting replication or spread during compatible infections.

There are a number of reports that virus infection of both compatible and incompatible hosts stimulates SA signaling. Markers for SA signaling (e.g., *PR-1*) (Volko et al. 1998), are up-regulated during systemic infections by several RNA viruses (Huang et al. 2005; Whitham et al. 2003). Callaway and associates (1996) reported that during *Cauliflower mosaic virus* (CaMV) infection of *Arabidopsis*, expression of *PR-1* and *PR-2* was enhanced, although resistance was not associated with necrotic lesions. The kinetics of activation was slower with compatible than incompatible host–virus combinations (Callaway et al. 1996; Dempsey et al. 1997). CaMV infection stimulated SA-dependent expression of *PR-1*, *PR-2*, and *PR-5* in susceptible *Arabidopsis* ecotypes (Laird et al. 2004; Love et al. 2005); however, neither exogenous application of SA nor an *NahG* transgene had any significant effect on virus accumulation (Love et al. 2005).

In a *Cucumber mosaic virus* (CMV)-susceptible *Arabidopsis* background, the SA-accumulating *ssi2* mutant exhibited increased resistance to the virus, but this was still evident in the mutant expressing *NahG* (Sekine et al. 2004). These authors also reported that application of SA to the leaves of wild-type *Arabidopsis* did not induce resistance to CMV, further evidence that defense against CMV is SA independent.

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In contrast, Chandra-Shekara and associates (2004) reported that application of SA to *Arabidopsis* enhanced resistance to CMV in a resistance gene-dependent manner, and Mayers and associates (2005) reported that, although exogenous application of SA induced resistance to CMV in *Arabidopsis*, it did not do so in squash. In a resistant *Arabidopsis* background, ethylene (ET), and SA also were reported to work synergistically against an avirulent isolate of CMV (Takahashi et al. 2002). Conclusions that can be drawn from these apparently conflicting reports are that the role of SA in plant defense signaling is not straightforward, and its efficacy is dependent on the host-virus combination.

We have shown that, in *Arabidopsis*, CaMV infection stimulates expression of markers for at least three defense signaling pathways (Love et al. 2005), each with different kinetics of activation. The effect of these responses on virus multiplication or spread was uncertain, although genetic knockout of the NADPH oxidase, which abolished the rapid systemic generation of H₂O₂ and the activation of ROS-responsive markers, did not significantly alter susceptibility to CaMV.

Mutants with defects in defense signaling have proven extremely valuable in studying defense responses for a variety of pathosystems. Mutant studies have elucidated specific aspects of plant defense during both compatible and noncompatible virus infection (Callaway et al. 1996; Chandra-Shekara et al. 2004; Kachroo et al. 2000; Love et al. 2005; Sekine et al. 2004). We previously have reported that accumulation of CaMV was reduced in the ET-signaling mutant *etr1-1*, but that the susceptibility of *NahG* was similar to the wild type. However, data from mutations at single loci are open to misinterpretation because the phenotypes are often pleiotropic—for example, *etr1-1* is deficient in H₂O₂ as well as ET perception (Desikan et al. 2005), and the extreme susceptibility of *NahG* to a variety of pathogens is at least partly attributable to the accumulation of catechol (Heck et al. 2003; van Wees and Glazebrook 2003). Consequently, single-mutant studies cannot identify unambiguously the involvement of signaling pathways; confirmation using several mutants (each affecting a different component of the pathway) is essential.

Here, we have expanded greatly the use of characterized mutants to unravel defense signaling associated with the compatible interaction between CaMV and *Arabidopsis*. We show that, although mutants with constitutively elevated levels of SA show enhanced resistance, mutants deficient in SA accumulation do not show enhanced susceptibility. It is likely that the resistance that we observe in SA-overexpressing mutants represents derepression of SA signaling by an inhibitory path-

way or SA induction of a complementary defense-response pathway. We also confirm and extend our recent report (Love et al. 2005) to show that ET-signaling mutants support reduced virus titers and impede long-distance movement.

RESULTS

Defense responses potentially limiting the multiplication and spread of CaMV in *Arabidopsis* were analyzed by infecting a comprehensive series of mutants that are affected in various aspects of SA and jasmonic acid (JA)/ET signaling (Table 1). Approximately 30 plants of each of the 13 genotypes were inoculated manually on a single leaf with 100 ng of purified CaMV. Plants were scored at 14 and 24 days postinoculation (dpi) for the symptoms of systemic infection, such as vein chlorosis in the expanded leaves, mosaics, and stunting and distortion of the leaves in the centre of the rosette. Real-time quantitative polymerase chain reaction (Q-PCR) was used to quantify virus DNA in plants showing systemic symptoms. We used the CaMV isolate CM4-184, which causes relatively mild symptoms in *Arabidopsis* (Cecchini et al. 1998), to facilitate identification of enhanced disease resistance phenotypes. However, parallel infections of mutants using the severe isolate Cabb B-JI gave no substantive differences in results. Therefore, we present only the data for CM4-184.

Susceptibility to infection of mutants with defects in SA signaling.

We first tested genotypes that are compromised in SA accumulation: *sid2-2*, *pad4-1*, *eds5-1*, and *NahG*. These show enhanced susceptibility to biotrophic bacterial and fungal pathogens and, therefore, were good candidates for enhanced susceptibility to CaMV. In general, no significant increases in susceptibility to infection were observed between any of the SA-deficient genotypes and wild-type Col-0 plants at either 14 or 24 dpi (Fig. 1A) although, in *NahG* at 24 dpi, leaf distortion and stunting were very severe compared with Col-0. In *NahG*, *sid2-2*, and *pad4-1*, levels of virus DNA in symptomatic plants did not differ significantly from levels in Col-0 at both time points. Unexpectedly, levels of virus DNA in *eds5* were moderately but significantly ($P < 0.05$) lower than in the wild type at both time points (Fig. 1B). Another mutant, *npr1*, which is compromised downstream of SA in the expression of members of the *PR-1* regulon (Cao et al. 1997), also accumulated amounts of virus DNA similar to the wild type (Fig. 1A and B).

CPR5 and *CPR1* act as negative regulators of SA biosynthesis (Bowling et al. 1997; Clarke et al. 2001). Loss-of-function

Table 1. Details of mutants tested for susceptibility to *Cauliflower mosaic virus* infection

Mutant allele	Gene ID	Wild-type protein encoded	Reference	Source ^a
<i>sid2-2</i>	At1g74710	Isochorismate synthase, required for salicylic acid (SA) biosynthesis	Wildermuth et al. 2001	F. Ausubel
<i>pad4-1</i>	At3g52430	Lipase-like protein, positive regulator pathogen-dependent SA signaling	Jirage et al. 1999	NASC
<i>eds5-1</i>	At4g39030	Multidrug resistance-domain protein, lipase-like protein, positive regulator pathogen-dependent SA signaling	Volko et al. 1998	NASC
<i>npr1-1</i>	At1g64280	Ankyrin repeat protein, required for <i>PR-1</i> activation	Cao et al. 1997	NASC
<i>cpr1-1</i>	Unmapped	Negative regulator of SA signaling, maps to complex R-like locus on Chr4	Bowling et al. 1994	X. Dong
<i>cpr5-2</i>	At5g64930	Membrane protein, negative regulator of pathogen-dependent SA signaling	Boch et al. 1998; Bowling et al. 1997	NASC
<i>cpr5 eds5</i>		Double mutant, <i>eds5-1</i> and <i>cpr5-1</i>	Clarke et al. 2000	X. Dong
<i>etr1-1</i>	At1g66340	Ethylene (ET) receptor, histidine kinase, strong dominant allele	Bleecker et al. 1988	NASC
<i>etr1-3</i>	At1g66340	ET receptor, histidine kinase, weak dominant allele	Roman et al. 1995	NASC
<i>ein2-1</i>	At5g03280	ET signaling intermediate, MAPK	Guzman and Ecker 1990	NASC
<i>eds4-1</i>	Unmapped	Reduced ISR (induced systemic resistance) in response to <i>Pseudomonas fluorescens</i> WCS417r, reduced <i>PDF1.2</i> expression, protein unknown	Ton et al. 2002	NASC
<i>eds8-1</i>	Unmapped	Reduced ISR in response to <i>P. fluorescens</i> WCS417r, reduced <i>PDF1.2</i> expression, protein unknown	Ton et al. 2002	NASC

^a Mutants were obtained from the sources shown: F. Ausubel, Harvard, Cambridge, MA, U.S.A.; NASC = Nottingham Arabidopsis Stock Centre, Nottingham, U.K.; X. Dong, Duke University, Durham, NC, U.S.A.

mutants accumulate high levels of SA constitutively and show enhanced resistance to biotrophic fungal and bacterial pathogens (Bowling et al. 1997; Clarke et al. 2000). Callaway et al. (1996) reported that symptoms of CaMV infection were delayed in *cpr1*. We found that *cpr1-1* and *cpr5-2* showed greatly enhanced resistance to CaMV (Fig. 1A and 1). Compared with the wild type, only approximately one-quarter to one-third the number of plants developed systemic symptoms at 14 and 24 dpi (Fig. 1A). Asymptomatic *cpr5* and *cpr1* plants accumulated levels of virus that were typically <1% that in symptomatic wild-type plants (Fig. 1B). Even in plants that did develop symptoms, levels of virus DNA were only approximately one-third that of the wild type (Fig. 1B). Although the *cpr5-2* allele is in a Nossen/Columbia hybrid background (Boch et al. 1998), the CaMV resistance of *cpr5-2* is not a consequence of the ecotypic background because virus DNA levels in wild-type No-0 actually were significantly higher than in Col-0 (Fig. 1B).

The *eds5* mutation, when introduced into a *cpr5* background, has been reported to abolish SA overproduction, although plants

retained elevated *PR-1* expression (Clarke et al. 2000). Also, in a *cpr5 eds5* double mutant, the *eds5* allele enhanced susceptibility to virulent isolates of *Pseudomonas syringae* and to avirulent isolates of *Peronospora parasitica* (Clarke et al. 2000, 2001). On this basis, we anticipated that *cpr5 eds5* might be more susceptible than *cpr5* to CaMV-infection. However, in *cpr5 eds5*, the proportion of symptomatic plants and virus DNA levels were essentially identical to that of *cpr5* (Fig. 1A and B).

To compare SA signaling responses in mutants and the wild type, we quantified the expression of *PR-1*, a reliable SA-responsive marker (Volko et al. 1998), in CaMV-infected plants. As we have reported previously (Love et al. 2005), *PR-1* transcripts rose in Col-0 as the infection progressed (Fig. 2A). In contrast, in the CaMV-resistant ecotype En-2 (Callaway et al. 1996), none of the plants developed symptoms and *PR-1* transcripts remained at a basal level (Fig. 2A). We next measured levels of *PR-1* transcripts in selected mutants at 14 dpi (Fig. 2B), a time at which levels of *PR-1* transcripts were strongly elevated in infected Col-0. *PR-1* expression was very low in

Fig. 1. Susceptibility to *Cauliflower mosaic virus* (CaMV) infection of wild-type, mutant, and transgenic *Arabidopsis* genotypes. **A**, Percentages of plants showing systemic symptoms—mosaics, vein chlorosis, stunting, or leaf distortion—at 14 days postinoculation (dpi) (gray bars) and 24 dpi (white bars). Results show mean values from three independent groups each comprising approximately 30 plants. Error bars show standard deviations (sd). **B**, CaMV DNA levels at 14 dpi (gray bars) and 24 dpi (white bars). Assays were carried out by real-time quantitative polymerase chain reaction as described in Materials and Methods. For each sample, tissue from a group of three plants was pooled. Values for each genotype are the means from six samples—three independent experiments each comprising two batches of pooled plants. Error bars show standard deviations. Tissue was taken only from plants showing systemic symptoms, except for *cpr1*, *cpr5*, and *cpr5 eds5*, where tissue was taken separately from pooled asymptomatic (indicated by A on the axis) and symptomatic (indicated by S) plants. Virus DNA levels (in arbitrary units) were normalized using 18S rDNA as an internal reference.

both infected and mock-inoculated *eds5*. *PR-1* transcripts were much more abundant in mock-inoculated *cpr5* and *cpr5 eds5* than in the corresponding wild type. However, in *cpr5*, CaMV infection stimulated a further threefold increase in transcript levels whereas, in *cpr5 eds5*, there was no such infection-dependent increase.

Susceptibility to infection of mutants with defects in ET signaling.

The *etr1-1* mutant showed reduced susceptibility to CaMV Cabb B-JI (Love et al. 2005). However, this allele of *etr1* is defective in H_2O_2 as well as in ET perception (Desikan et al. 2005), raising the possibility that the susceptibility phenotype might be a consequence of deficiencies in ROS signaling. Therefore, we infected a further two ET-signaling mutants—*etr1-3*, an *etr1* allele which is unaffected in H_2O_2 perception (Desikan et al. 2005), and *ein2-1*—plus two additional mutants, *eds4* and *eds8*, that originally were identified as being defective in induced systemic resistance (Ton et al. 2002). EDS4 is involved in cross-talk between SA-dependent and ET/JA-dependent defense signaling pathways, whereas EDS8 functions as a positive regulator of ET/JA-dependent defense (Ton et al. 2002). In both *etr1-1* and *etr1-3*, and in *ein2*, the appearance of systemic symptoms was delayed by 2 days compared with the wild type and, in all three mutants, symptoms were much milder than in Col-0. Although none showed statistically significant differences in the proportions showing symptoms at 24 dpi ($P > 0.05$) (Fig. 1A), virus accumulation in all three mutants at both 14 and 24 dpi was very significantly lower than in the wild type (Fig. 1B). The proportions of *eds4* and *eds8* showing symptoms and virus titers at both 14 and 24 dpi also were significantly lower than in the wild type (Fig. 1A and B).

Involvement of the alternative oxidase (Aox)–associated antiviral pathway in defense.

An Aox-associated pathway has been implicated in antiviral defense (Murphy et al. 1999) and treatment with salicyl-hydroxamic acid (SHAM), which acts inter alia as an inhibitor of Aox activity, antagonizes SA-induced resistance to TMV in a susceptible tobacco cultivar (Chivasa et al. 1997). We applied SHAM to Col-0 and *cpr5* for 1, 2, or 3 successive days prior to manual inoculation with CaMV CM4-184. SHAM had no effect on the proportion of symptomatic Col-0 plants at 18 dpi; however, treating *cpr5* with 1 mM SHAM for three successive days

increased the proportion of symptomatic plants from 16 to 62%, close to the 63% in SHAM-treated wild-type plants.

Because three successive daily applications of 1 mM SHAM reversed the resistant phenotype of *cpr5*, we applied it to *cpr5*, *eds5*, *cpr5 eds5*, *cpr1 etr1-1*, and *ein2*, inoculated the plants with CaMV CM4-184, and compared the development of symptoms over the period up to 18 dpi (Fig. 3). Systemic symptoms first were visible at approximately 11 dpi. With Col-0, *etr1-1 ein2*, and *eds5*, we observed no obvious differences between the three different genetic backgrounds in the proportions of symptomatic plants (Fig. 3A and B; for clarity, the results for *ein2*, which are similar to those for *etr1-1*, are omitted). However, compared with buffer-treated controls, SHAM treatment resulted in a modest increase in the proportion of symptomatic plants at 12 dpi for all four genotypes, although not at other time points. Analysis of variance indicated that there were no significant differences between genotypes ($P > 0.05$), but that the difference between SHAM and buffer treatment in the proportion of symptomatic plants at 12 dpi was significant ($P < 0.05$). The increased proportion of symptomatic plants early in the infection suggests that SHAM treatment might be accelerating virus spread slightly. Buffer-treated *cpr1* was much less susceptible to infection than Col-0, and treatment with SHAM resulted in only a slight increase in susceptibility, which was not significant ($P > 0.05$) (Fig. 3C). For *cpr5 eds5* and *cpr5*, the proportion of symptomatic plants was very much lower than for Col-0 in the buffer-treated group. However, in contrast to the other genotypes, SHAM treatment substantially although not completely reversed the resistant phenotype, increasing the proportion of plants developing symptoms by three- to fivefold (Fig. 3D). The central leaves of SHAM-treated *cpr5* and *cpr5 eds5* were severely stunted and distorted, demonstrating that extensive systemic spread had occurred. In neither CaMV-infected nor mock-inoculated *cpr5* did SHAM treatment affect the development of necrotic lesions.

On the basis that SHAM is an Aox inhibitor, Carr and co-workers identified a potential role for Aox in antiviral defense (Murphy et al. 2001). In order to determine whether CaMV infection might be associated with changes in expression of the SA-inducible isoform *AOX1A* in *Arabidopsis* (Saisho et al. 1997), we determined the effect of CaMV infection on transcript levels in Col-0 and four mutants, *ein2-1*, *etr1-1*, *cpr1*, and *cpr5*. Transcripts were quantified by real-time reverse-

Fig. 2. A, Time course of *PR-1* expression in a susceptible and a resistant ecotype. Tissue was harvested from *Cauliflower mosaic virus* (CaMV)-infected plants at various times after inoculation. Transcript levels in Col-0 (◆) and Ein-2 (□) were measured by real-time reverse-transcription polymerase chain reaction (RT-PCR) and were normalized using *ACT2* as an internal standard. Transcripts levels (in arbitrary units) show mean $\pm$ standard deviation (sd) for three samples, each representing pooled tissue from three individual plants. **B**, Expression of *PR-1* in wild-type and mutant *Arabidopsis*. Tissue was harvested at 14 days postinoculation. Transcript levels in CaMV-infected (white bars) and mock-inoculated (gray bars) plants were measured by real-time RT-PCR. Transcripts levels (in arbitrary units normalized to *ACT2*) show mean $\pm$ sd for three samples, each representing pooled tissue from three individual plants; *levels of *PR-1* transcripts in uninfected *eds5* plants were <0.01 arbitrary units.

transcription (RT)-PCR in mock- and CaMV-inoculated (symptomatic only) plants at 14 dpi (Fig. 4). *AOX1A* transcripts were approximately four times more abundant in infected than in mock-inoculated Col-0. In mock-inoculated *ein2-1*, *etr1-1*,

and *cpr1*, basal levels did not differ significantly from the wild type and, as in Col-0, virus infection stimulated an increase of greater than threefold. In mock-inoculated *cpr5*, *AOX1A* transcripts were more than five times more abundant than in the wild type. Enhanced expression of *AOX1A* appears to be a consistent phenotypic feature of *cpr5* because transcript levels in uninfected *cpr5* were at least threefold greater than in the wild type at both the two-leaf stage and in older rosette plants (data not shown). Infection did not stimulate a further increase in transcript levels in *cpr5*; indeed, levels were slightly lower than in mock-inoculated controls grown in parallel, although the differences were not statistically significant ($P > 0.05$).

Application of AA, which blocks mitochondrial electron transport between complex III and IV and increases electron flow through Aox (Moller 2001), stimulates resistance to several viruses (Wong et al. 2002), including CaMV (Love et al. 2005). To test whether this resistance might require SA or ET signaling downstream of Aox, we determined the effect of AA on wild-type, SA-deficient, and ET-insensitive genotypes. When AA was included in the inoculum, fewer than 12% of wild-type, *sid2-2*, *NahG*, or *etr1-1* plants had developed symptoms at 24 dpi, compared with more than 80% of controls (no AA) (Fig. 5). Therefore, the ability of AA to induce resistance did not differ between mutant and wild-type genotypes. As a further control, we examined the effect of Rotenone, which blocks mitochondrial electron transport between complex I and II and, hence, does not stimulate Aox activity (Moller 2001). Treatment with 10 to 200 μ M Rotenone had no significant effect on the accumulation of CaMV in any of the genotypes, even at the highest concentrations used (data not shown).

Fig. 3. Effect of salicyl-hydroxamic acid (SHAM) treatment on susceptibility in wild-type and mutant *Arabidopsis*: **A**, *etr1-1*; **B**, *eds5*; **C**, *cpr1*; and **D**, *cpr5* and *cpr5 eds5*. To facilitate comparison, the Col-0 results are reproduced in each panel. Plants were treated with 1 mM SHAM or buffer for three successive days before manual inoculation with *Cauliflower mosaic virus* CM4-184, as described in Materials and Methods. The percentages of plants showing systemic symptoms were scored over the period from 0 to 18 days postinoculation. Open symbols represent buffer-treated controls and closed symbols represent SHAM-treated plants. Symbols for each individual genotypes are indicated in the figure legends. Results show mean $\pm$ standard deviation for two independent experiments, each comprising approximately 30 plants of each genotype.

Fig. 4. Expression of *AOX1A* in wild-type and mutant *Arabidopsis*. Tissue was harvested at 14 days postinoculation. Transcript levels in *Cauliflower mosaic virus* (CaMV)-infected (white bars) and mock-inoculated (gray bars) plants were measured by real-time reverse-transcription polymerase chain reaction. Transcript levels (in arbitrary units normalized to *ACT2*) show mean $\pm$ standard deviation for three samples, each representing pooled tissue from three individual plants.

Virus replication and movement in mutant and wild-type plants.

To determine whether resistance in *cpr1*, *cpr5*, and *etr1* was a consequence of reduced virus replication, a reduction in cell-to-cell movement, or reduced systemic spread, we monitored virus movement using a recombinant CaMV engineered to express green fluorescent protein (GFP) as a free protein. Manual inoculation with CaMV-GFP was unsuccessful; therefore, infection was accomplished by bombardment with gold particles coated with virus DNA.

We first optimized the bombardment conditions using DNA from CaMV isolate Cabb B-JI, the parental isolate of CaMV-

Fig. 5. Effect of Antimycin A (AA) treatment on mutants: Plants were inoculated manually with *Cauliflower mosaic virus* Cabb B-JI and the percentage showing symptoms was scored at 24 days postinoculation. The inoculum included in addition to virus was 50 μ M AA + 0.1% dimethyl sulfoxide (DMSO) (gray bars) or 0.1% DMSO only (white bars). Results are mean values $\pm$ standard deviation from two groups of 30 to 40 plants of each genotype.

GFP. With Col-0, 100% of plants developed chlorotic local lesions by 14 dpi, and the severe stunting and distortion of the central emergent leaves by 21 dpi demonstrated that bombardment was a very efficient inoculation technique (Fig. 6A). In *etr1-1*, systemic infection rates also were 100%, although symptoms were much milder (Fig. 6B). All of the *cpr5* and *cpr1* plants developed local lesions on the bombarded leaves; however, unlike Col-0, only approximately half of them progressed to systemic symptom development by 24 dpi. In *cpr1*, local lesions were chlorotic and systemic symptoms were always very mild (Fig. 6C). In *cpr5*, local lesions showed evidence of necrosis and those plants that developed a systemic infection progressed to necrosis and death, typically by 28 dpi (Fig. 6D). We tested whether the concentration of virus DNA in the inoculum might be contributing to the higher proportion of *cpr1* and *cpr5* plants developing systemic symptoms when infected by bombardment. However, even when we reduced the amount of virus DNA from 27.5 to 0.275 ng/plant, lesion numbers, sizes, and the proportions of plants that developed systemic symptoms were unaffected in all backgrounds (data not shown).

With CaMV-GFP, all Col-0 plants showed visible chlorotic local lesions by 14 dpi and systemic symptoms by 21 dpi, although these appeared approximately 4 days later and were milder than with Cabb B-JI. All *cpr1* and *cpr5* also developed visible local lesions (becoming necrotic in the case of *cpr5*); however, by 21 dpi, fewer than 20% of the plants showed any symptoms of systemic infection, and then usually on only a single leaf. In CaMV-GFP, open reading frame (ORF) II was replaced by the coding sequence for a modified GFP. We do not believe that the low pathogenicity of CaMV-GFP results from lack of the ORF II product, because CaMV CM4-184, which also fails to produce a functional P2 (Delseny and Hull 1983), is fully infectious when inoculated manually onto *Arabidopsis* and, like Cabb B-JI, CM4-184 partially overcame resistance in *cpr1* and *cpr5* when inoculated by bombardment (data not shown).

Fig. 6. Symptom development in wild-type and mutant *Arabidopsis* plants 21 days after bombardment with (top row) gold particles coated with *Cauliflower mosaic virus* (CaMV) Cabb B-JI DNA or (bottom row) mock-inoculated, gold only. Each panel shows a cell (container) with two plants. **A**, Infected Col-0; **B**, infected *etr1-1*; **C**, infected *cpr1-1*; **D**, infected *cpr5-2*; **E**, uninfected Col-0; **F**, uninfected *etr1-1*; **G**, uninfected *cpr1-1*; and **H**, uninfected *cpr5-2*. Photographs were taken at 21 days postinoculation (dpi) except for *cpr5-2*, which is at 28 dpi.

We carried out two independent experiments in which we bombarded five plants of each genotype with CaMV-GFP, and examined at least three bombarded leaves and three post-bombardment emergent leaves from each plant using confocal microscopy. In wild-type plants, foci of approximately 10 strongly fluorescing epidermal cells were visible by 10 dpi, typically approximately 5 or 6 per leaf. These fluorescent areas, which coincided with the chlorotic local lesions, expanded with time and, by 20 to 21 dpi, epidermal cells within and around the periphery were fluorescing strongly (Fig. 7A and B). We routinely observed strong GFP fluorescence in cells within minor veins leading away from the lesion (Fig. 5A), and cells in the midveins of bombarded leaves always were strongly fluorescent, particularly near the petiole (Fig. 7C). Presumably, virus moves out of the primary lesions, progressively infecting cells within the vascular system.

Bombarded leaves from *etr1-1* appeared similar to those in the wild type, with epidermal and associated vascular cells showing strong GFP fluorescence (Fig. 7D). Bombarded leaves

from *cpr1* contained fewer and smaller local lesions than the wild type or *etr1-1* (Fig. 7E, sizes can be compared between E and A). We sometimes observed GFP fluorescence in cells associated with adjacent minor veins (Fig. 7E).

Bombarded leaves from *cpr5* contained strongly fluorescing local lesions. As with *cpr1*, these were fewer and smaller than in wild-type plants. However under bright-field microscopy, the lesions in *cpr5* were much more distinct than in any of the other genotypes (Fig. 7F, as compared with B). Cells within the lesions typically lacked the usual fluorescent cellular structures (e.g., nuclei or cytoplasmic strands) and fluorescence associated with the cell periphery was punctate rather than continuous (compare Fig. 7F with B and G with A). The *cpr5* plants bombarded with DNA from unmodified Cabb B-JI developed similar fluorescent lesions (not shown), presumably as a consequence of the autofluorescence induced in necrotic tissue. We did not observe such fluorescence with Cabb B-JI in any other genotype, or in *cpr5* bombarded with gold only. Therefore, CaMV infection triggers cell death in *cpr5*.

Fig. 7. Confocal microscope images from plants infected with CaMV-GFP, a recombinant virus engineered to express green fluorescent protein (GFP). All images are at the same magnification and the white scale bar indicates 500 μ m. The excitation wavelength was 488 nm. Emission in the GFP channel (505 to 530 nm) is rendered in green and emission at wavelengths 560 to 615 nm is rendered in red. **A, B,** and **F,** GFP fluorescence channel only; **C** through **E** and **G** through **L,** both fluorescence channels. **A,** Col-0 bombarded leaf, 21 days postinoculation (dpi), local lesions with spread to minor vasculature (white arrows); **B,** the same image as A but with bright field superimposed in black and white; **C,** Col-0 bombarded leaf, 21 dpi, midvein near petiole (white arrow); **D,** *etr1-1* bombarded leaf, 16 dpi, local lesion; **E,** *cpr1-1* bombarded leaf, 16 dpi, local lesion; **F,** *cpr5-2* bombarded leaf 16 dpi, autofluorescent local lesion, GFP channel only with bright-field image superimposed in black and white; **G,** the same image as in panel F, without bright field but with both 505- to 530- and 560- to 615-nm channels; **H,** *cpr5-2* bombarded leaf, 20 dpi, autofluorescent local lesion (orange arrows) surrounded by living infected epidermal cells (green arrows) and infected vasculature (white arrows); **I,** Col-0 unexpanded newly emergent leaf, 21 dpi, systemic infection; **J,** *etr1-1* unexpanded newly emergent leaf, 21 dpi, systemic infection confined to midvein (white arrow); **K,** *cpr1* unexpanded newly emergent leaf, 21 dpi, systemic infection confined to a small number of vascular cells (white arrow); and **L,** *cpr5* unexpanded newly emergent leaf, 28 dpi, infection confined to the midvein and one subsidiary vein (white arrows).

To determine the extent to which autofluorescence might be a source of the emission in the GFP channel, we examined CaMV-GFP-infected leaves using a second set of emission filters with the range 560 to 615 nm. These exclude emission from modified (M)-GFP but include part of the broad autofluorescence emission spectrum. In *cpr5*, areas of fluorescence at 560 to 615 nm and 505 to 530 nm (Fig. 7G, red and green, respectively) usually were coincident and fell within the location of the lesion as observed under bright-field microscopy (Fig. 7F and G). With none of the other *Arabidopsis* genotypes did we detect any fluorescence from 560 to 615 nm; emission was confined exclusively to the 505- to 530-nm GFP channel (compare Fig. 7G with D and E). Therefore, in *cpr5*, the major source of emission in CaMV-GFP lesions almost certainly was autofluorescence. However, in approximately 10 to 20% of the necrotic lesions, we observed zones of authentic GFP fluorescence, intact cells around the periphery of the lesion and in the adjacent vasculature where emission was restricted to wavelengths between 505 and 530 nm (Fig. 7H). The presence of live cells containing replicating virus showed no obvious correlation with time after inoculation, suggesting that we were not observing a particular stage in lesion development.

We examined post-bombardment emergent leaves for evidence of systemic virus movement. In Col-0, by 20 dpi, almost all the fully expanded (>6 mm long) and unexpanded (<3 mm long) leaves exhibited strong GFP fluorescence (Fig. 7I). In contrast, in *etr1-1*, these areas of fluorescence, particularly in unexpanded leaves, were much less extensive than in Col-0. Many of the vascular cells were nonfluorescent and, unlike in Col-0, spread of virus from the vascular system into adjacent areas of the leaf typically was limited or absent (Fig. 7J, as compared with I). In *cpr1*, nearly all of the nonbombarded leaves were asymptomatic. We rarely were able to identify infected cells in unexpanded leaves and, even in the occasional symptomatic expanded leaf, fluorescence was limited to small groups of cells (Fig. 7K). In *cpr5*, we rarely detected systemic infection by 21 dpi although, by 28 dpi, limited parts of the vascular system within the occasional unexpanded leaf showed authentic GFP fluorescence (Fig. 7L, as compared with J). Thus, in most *cpr1* and *cpr5* plants, systemic movement of CaMV-GFP was undetectable and, in the rest, virus spread was confined to a small number of cells in the occasional leaf.

DISCUSSION

We have reported previously that three distinct defense-related signaling pathways are activated in the susceptible host *Arabidopsis* in response to CaMV infection (Love et al. 2005). Here, we have undertaken a study using a comprehensive set of mutants to establish the importance of each pathway in mounting defense against CaMV, to define critical components of the signaling pathways, and to further our understanding of defense initiation.

SA-dependent defense responses and the role of SA-independent signaling.

None of the genotypes with deficiencies in either SA accumulation or signaling showed enhanced susceptibility to CaMV. However, the two mutants that constitutively overexpress SA, *cpr1* and *cpr5*, showed a robust, enhanced resistance phenotype that was exhibited against two different CaMV isolates and, in bombarded plants, was maintained over a 100-fold range of virus DNA concentration. Callaway and associates (1996) reported a 2-day delay in symptom appearance and reduced severity in CaMV-infected *cpr1*. Possibly their use of agroinoculation to achieve infection might be responsible for their relatively modest degree of resistance. CaMV infection stimu-

lates expression of SA-responsive markers (Laird et al. 2004; Love et al. 2005); therefore, we assume that SA signaling is activated. However, the absence of an enhanced susceptibility phenotype in SA-deficient plants suggests that it is ineffective during CaMV infection of wild-type *Arabidopsis*.

In addition to elevated SA levels, *cpr5* shows an SA-independent lesion-mimic phenotype (Bowling et al. 1997). Noting that the SA-dependent pathway may be ineffective in wild-type *Arabidopsis*, an explanation for the resistance in *cpr5* might be a hypersensitive response (HR) that halts the spread of virus from local lesions. However, *Arabidopsis* must possess other mechanisms besides HR, because *cpr1*, which does not develop necrotic lesions, is equally resistant to CaMV.

The resistant phenotype of the *cpr5 eds5* double mutant was unexpected. The presence of a mutant *eds5* allele restores the sensitivity of *cpr5* to virulent *Pseudomonas syringae* and enhances susceptibility to avirulent *Peronospora parasitica* (Clarke et al. 2000). Although Clarke and associates (2000) found that uninfected *cpr5 eds5* contained only very low levels of SA, levels of *PR-1* transcripts were much greater than in wild-type plants. *HRT*-dependent resistance to TCV requires both *EDS1* and *EDS5* (Chandra-Shekara et al. 2004), although it may be significant that introducing a mutant *eds5* allele into a resistant background compromised resistance but not HR.

Uninfected *cpr5 eds5* accumulated high levels of *PR-1* transcripts. Clarke and associates (2000) did not compare the effect of infection on levels of *PR-1* in the mutants. We found that, in the single but not the double mutant, CaMV infection stimulated a further increase in *PR-1* abundance. A possible explanation for CaMV resistance in *cpr5* and *cpr5 eds5* is the involvement of an EDS5-independent pathway, perhaps related to the induction of cell death. The very low levels of *PR-1* transcripts in infected *eds5* and the absence of virus-dependent upregulation in *cpr5 eds5* suggest that CPR5 may be signaling via two distinct pathways. One presumably involves EDS5 and is required for the virus-dependent component of the activation of *PR-1*; the other bypasses EDS5 and appears to be responsible for virus resistance in the *cpr5* mutant, cell death, and elevated *PR-1* expression in the uninfected double mutant.

Resistance requires the SHAM-sensitive antiviral defense pathway in *cpr5* but not in *cpr1*.

AA inhibits mitochondrial complex III, stimulating electron flow through Aox (Moller 2001). Carr and co-workers have proposed a model in which elevated SA levels stimulate a virus-specific defense pathway (Gilliland et al. 2003; Singh et al. 2004) via alterations in electron flow through Aox, which is a negative regulator of ROS. AA treatment promotes resistance to CaMV in wild-type *Arabidopsis* (Love et al. 2005) and we show here that SHAM treatment results in a statistically significant, albeit modest, acceleration in virus spread in wild-type and several mutant genotypes. Therefore, the Aox-associated pathway must be able to promote an effective defense response upon activation (although the extent to which it is activated during infection in wild-type plants is unclear).

In *cpr5* and *cpr5 eds5*, treatment with SHAM restored susceptibility, albeit not to wild-type levels; whereas, in *cpr1* and *etr1-1*, resistance or reduced susceptibility were insensitive to SHAM. *AOX1A* transcripts in uninfected *cpr1*, *etr1*, *ein2*, and *eds5* plants were at a basal level, but increased in response to CaMV infection. Transcript levels in uninfected *cpr5-2* already were elevated and infection had no additional effect. In *cpr5*, enhanced *AOX1A* expression might be linked to the development of necrotic lesions, rather than to increased levels of SA. Overexpression of Aox has been reported to affect resistance or the development of necrosis during infection by TMV (Murphy et al. 2004; Ordog et al. 2002) and we cannot discount

the possibility that, in *cpr5*, resistance to CaMV might be linked to cell death. Both SA and oxidative stress have been reported to upregulate transcription of the alternative oxidase gene (Buchanan-Wollaston et al. 2005; Norman et al. 2004; Polidoros et al. 2005). Therefore, it is surprising that *AOX1A* transcripts were low in uninfected *cpr1*, because SA levels are elevated in this mutant (Clarke et al. 2001).

ET signaling and pathogenicity.

We can now exclude the possibility that the reduced susceptibility of *etr1-1* (Love et al. 2005) is linked to the altered H₂O₂ perception of this allele (Desikan et al. 2005), because *etr1-3*, *ein2-1*, and the ET/JA-mediated defense mutants *eds4* and *eds8* also showed significantly reduced susceptibility. Clarke and associates (2001) identified a role for ET in SA-dependent NPR1-independent responses. We have demonstrated previously that transgene-mediated expression of CaMV protein P6, an important virus-encoded pathogenicity determinant, results in a strong ET-insensitive phenotype (Geri et al. 2004). Signaling through the ET/JA- and SA-mediated defense pathways is mutually antagonistic (Kunkel and Brooks 2002; Wiermer et al. 2005). A plausible explanation for the reduced virus susceptibility of ET mutants is that removing ET signaling alleviates repression of SA signal transduction, thereby priming the plants for defense.

Mutants *cpr5*, *cpr1*, and *etr1-1* are compromised in long-distance virus movement.

We observed no obvious differences between the wild type and mutants in the GFP fluorescence of cells within local lesions. Therefore, replication appears not to be significantly compromised in these mutants. Fluorescent local lesions were somewhat smaller in the mutants; therefore, we cannot exclude a reduction in cell-to-cell movement. The most obvious difference between the wild type and mutants, particularly *cpr1* and *cpr5*, was in long-distance movement of virus. In most plants, CaMV-GFP failed to spread systemically and, in the rest, systemic movement was limited to small groups of cells in occasional leaves. For the virus to move systemically, it must be loaded into the vascular system from cells within the primary lesions, translocated through the phloem, and subsequently unloaded at distant susceptible sites. We observed GFP fluorescence in cells of the vasculature leading away from local lesions in both resistant mutants and the wild type. Thus, the target may be at the point of vascular exit rather than entry.

SA treatment has been reported to restrict long-distance movement of CMV in both tobacco and *Arabidopsis* (Mayers et al. 2005). In contrast, SA-dependent resistance to CMV in squash (Mayers et al. 2005) and to TVCV in *Arabidopsis* (Wong et al. 2002) appears to act at the level of virus replication. Therefore, antiviral defense responses may be capable of targeting replication or movement, depending on the virus–host combination (Singh et al. 2004). In *Nicotiana glutinosa*, CMV protein 2b antagonizes both RNA silencing and SA-mediated defense against CMV and TMV (Ji and Ding 2001), establishing a link between SA signaling and silencing, an important defense mechanism against virus infection (Baulcombe 2004), and a recent publication (Alamillo et al. 2006) suggests that SA may act as an enhancer of RNA-silencing in tobacco during infection by *Plum pox potyvirus*. Silencing is most likely initially enacted locally, and a mobile silencing signal then is translocated systemically (Schwach et al. 2005). This provides a plausible explanation for local inhibition of replication with tobamoviruses but inhibition of long-distance movement with cucumo- and caulimoviruses.

Inhibitor studies have identified a potential role for SA signaling in defense responses during compatible virus infections,

although such studies can lead to conflicting conclusions (Mayers et al. 2005; Sekine et al. 2004). SA-deficient *Arabidopsis* mutants do not exhibit enhanced susceptibility to CaMV. Thus, SA-dependent responses may be ineffective against CaMV infection in wild-type plants. However, when primed prior to infection, for example in *cpr* mutants, or following treatment with chemical elicitors, they are capable of inhibiting long-distance virus movement. The possibility that SA-dependent defense responses may act synergistically with RNA silencing (Alamillo et al. 2006) provides a plausible mechanism for the enhanced resistance of *cpr* mutants to CaMV. Certainly, CaMV elicits a vigorous SA signaling response in wild-type *Arabidopsis*, in which *PR-1* transcripts reach levels comparable to those in plants infected with avirulent *Pseudomonas syringae* (Laird et al. 2004). A further intriguing possibility is that, despite this, the virus may encode a suppressor of defense or silencing that allows it to evade the consequences of increased SA levels.

MATERIALS AND METHODS

Virus isolates and infection.

CaMV isolates CM4-184 and Cabb B-JI (Al Kaff and Covey 1995; Delseny and Hull 1983) were maintained and propagated in turnip (*Brassica rapa-rapifera* cv. Just Right) as described (Cecchini et al. 1998). Virus DNA was prepared as described (Al Kaff and Covey 1994). CaMV-GFP, a recombinant virus that expresses GFP, was a gift from D. Turner and S. N. Covey (John Innes Centre, Norwich, U.K.). CaMV-GFP, which expresses GFP as a free protein in the cytosol, was constructed by substituting ORFII of Cabb B-JI with a modified M-GFP5 (Chalfie et al. 1994). Full details of the construction and properties of CaMV-GFP will be published elsewhere. The full-length CaMV-GFP DNA sequence was inserted into pBluescript using the unique *SalI* site at nucleotide 4,831 of the Cabb B-JI sequence, and was maintained and propagated as plasmid DNA.

Arabidopsis plants were grown in compost in a controlled environment at a temperature of 22°C. Light was provided by Osram “Warm White” fluorescent tubes at an intensity of 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 10 h per day. Following the emergence of the first true leaves, *Arabidopsis* seedlings were inoculated manually on single leaf with 2 μl of water containing 100 ng of purified virus, as described by Cecchini and associates (1998).

For inoculation by particle bombardment, *Arabidopsis* was grown in 40-by-40-mm cell-tray inserts with two plants to each cell. Plants at the eight-true-leaf stage were inoculated with a Helios PDS 1000He particle gun (Bio-Rad Laboratories, Hemel Hempstead, U.K.) using a protocol modified from that of Sanford and associates (1993). Aliquots (25- μl) of 1.0 μm gold particles at a concentration of 40 mg/ml were suspended by sonication and mixed with 25 μl of H₂O and 5 μl of virus DNA (118 $\mu\text{g/ml}$). Then, 50 μl of 2.5 M CaCl₂ and 20 μl of 0.1 M spermidine were added, and the tube was incubated at room temperature for 3 min. The particles were precipitated by a 3-s centrifugation at 13,000 rev min⁻¹ in a bench-top centrifuge, resuspended in 150 μl of 100% ethanol, precipitated again by centrifugation, and resuspended in 85 μl of 100% ethanol. Each cell, containing two plants, was bombarded with 8 μl of the DNA-coated gold suspension using a 650 pound per square inch rupture disk.

Treatment with inhibitors or elicitors of defense.

A hand-held mister was used to spray plants with 1 mM SHAM in 10 mM Na phosphate buffer, pH 7.0, at 24-h intervals for 1, 2, or 3 days before inoculation. AA or rotenone

Table 2. Primer sets used in real-time quantitative polymerase chain reaction

Target	Forward primer	Reverse primer	Amplicon (bp)
<i>Cauliflower mosaic virus</i>	AGCGGTCAAAATATTGCTTA	AACCTACCGTATGCTAGATTACCT	141
Genomic 18S rDNA	CGTGATCGATGAATGCTACC	GGGGTTTGTTCACGTATTA	199
<i>PR-1</i>	TCAGTGAGACTCGGATGTG	CCTGCATATGATGCTCCTT	191
<i>AOX1A</i>	CTGGAGCTTCCTTTAGTTCA	ACATTGAGAATGTTCTCTGCT	174
<i>ACT2</i>	CTAAGCTCTCAAGATCAAAGGCTTA	ACTAAAACGCAAAACGAAAGCGGTT	218

treatment was accomplished by including 50 μ M AA or 10 to 200 μ M rotenone, respectively, in 0.1% dimethyl sulfoxide (DMSO) for manual inoculation (Love et al. 2005). Control plants were inoculated with virus in 0.1% DMSO.

***Arabidopsis* mutants and transgenic lines.**

Wild-type *Arabidopsis* Col-0 and No-0 originally were obtained from Nottingham Arabidopsis Stock Centre (Nottingham, U.K.). *NahG* transgenic line B6 was a gift from Novartis (Greensboro, NC, U.S.A.). Sources and other details of the *Arabidopsis* mutants used are given in Table 1. All mutants were in a Columbia background except *cpr5-2*, which were in a Nossen/Columbia background.

Assay of CaMV DNA.

DNA was purified and CaMV DNA was quantified by real-time Q-PCR as described by Love and associates (2005). For each sample, approximately 50 mg of leaf tissue was harvested from three plants and pooled. DNA was purified with a Nucleospin kit (Clontech, Oxford, England) according to the manufacturer's instructions, but with an additional step of digestion with proteinase K (Cecchini et al. 2002). DNA concentration was measured fluorometrically using PicoGreen double-stranded DNA quantitation reagent (Molecular Probes, Eugene, OR, U.S.A.) according to the manufacturer's protocol.

Q-PCR was carried out in a Stratagene MX4000 Real Time thermocycler (Stratagene, Amsterdam, The Netherlands) using Stratagene Brilliant kits according to the manufacturer's protocols. Each reaction contained 100 pg of total DNA. PCR conditions were 10 min at 95°C, then 40 cycles of 30 s at 95°C, 30 s at 55°C, and 60 s at 72°C. Primers for CaMV Cabb B-JI genomic DNA and for the *Arabidopsis* gene encoding 18S rRNA (used as an internal reference standard) have been described previously (Love et al. 2005) and are shown in Table 2.

For each sample, duplicate or triplicate sets of parallel reactions were carried out. Threshold cycle values were determined from the SyBr Green fluorescence using software provided by Stratagene. Regression lines were generated and amplification products quantified using an external standard, which was total DNA extracted at 21 dpi from a batch of *Arabidopsis* Col-0 plants infected with CaMV Cabb B-JI (Love et al. 2005). Analysis of the data and calculation of the amounts of CaMV DNA in each sample were carried out as described previously (Love et al. 2005). Normalized amounts of CaMV DNA are expressed in arbitrary units relative to the level in the external standard. A value of 1.0 is equivalent to an average of approximately 3,000 copies of the CaMV genome per diploid *Arabidopsis* genome.

Assay of transcripts.

Transcript levels were quantified by real-time RT-PCR using the Stratagene MX4000 Real-Time thermocycler as described by Love and associates (2005). RNA was extracted from approximately 50 mg of tissue as described previously (Cecchini et al. 1997; Love et al. 2005). RNA samples (50 ng) were transcribed to cDNA in 20- μ l reactions using Qiagen Sensiscript kits (Qiagen, Crawley, U.K.) according to the manufacturer's proto-

col. cDNAs were quantified using Stratagene Brilliant Kits as described above for the assay of CaMV DNA, but with minor modifications. Each 25- μ l reaction contained 0.2 μ l of cDNA, and PCR conditions were similar to those for virus DNA except that the annealing was carried out at 60°C. Reactions were carried out in triplicate for each biological sample, which comprised cDNA derived from the pooled RNA from three individual plants. Primers for *PR-1* (At2g14610), *AOX1A* (At3g22370), and *ACT2* (At3g18780) are shown in Table 2. *ACT2* was chosen as an internal reference because we previously have shown that it is expressed in a wide variety of cell types in *Arabidopsis* (Laval et al. 2002) and, from microarray data, that expression of *ACT2* appears to be broadly constitutive (V Laval, J. Laird, P. Armengaud, and J. J. Milner, *unpublished results*). Regression lines were generated using, as external standards, plasmid DNA containing full-length cDNA sequences of *PR-1*, *AOX1A*, and *ACT2*. Values for the concentration of the appropriate transcript cDNA were calculated by normalizing the raw values using the concentration of *ACT2* as an internal reference, as described by Love and associates (2005).

Fluorescence microscopy.

Replication and spread of CaMV-GFP was followed using a Zeiss LSM510 confocal microscope. For GFP fluorescence, the excitation wavelength was 488 nm and the emission filters were set for the range 505 to 530 nm. To discriminate between authentic GFP fluorescence and autofluorescence, a second emission channel was set for the range 560 to 615 nm.

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