The plant defense response to cucumber mosaic virus in cowpea is elicited by the viral polymerase gene and affects virus accumulation in single cells

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Resistance to infection in cowpea by strains of cucumber mosaic virus (CMV) involves a local, hypersensitive response (HR) and a localization of infection. These responses can be separated by mutation at two sites (nucleotides 1978 and 2007, in codons 631 and 641) in the CMV 2a polymerase gene. Changes to both sites of a restricted strain allow systemic infection without an HR and increase the accumulation of both the 2a protein and viral RNA in protoplasts, while changing position 1978 alone results in a systemic infection, a systemic HR, and an increase in viral RNA accumulation in protoplasts. It is suggested that the inhibition response observed in protoplasts, where an HR does not occur, leads to localization of infection in whole plants and that different plant genes are involved in eliciting the HR and the localization response.

Keywords: cucumber mosaic virus/hypersensitive response/plant defense response/plant protoplasts/plant virus replication

Introduction

Resistance to infection by plant viruses operates at various levels (reviewed by Fraser, 1990). Some resistance mechanisms block replication, some interfere with either local (cell-to-cell) or vascular (leaf-to-leaf) movement, and some induce a hypersensitive response (HR), related to programmed cell death (for review, see White and Antoniwi, 1991; Jones and Dangl, 1996). The HR usually results in localization of the virus to the site of inoculation and cells surrounding the initially infected cells. The *N* and *N'* genes in tobacco are the two best-characterized systems, in which an HR is induced by tobacco mosaic virus (TMV) (reviewed by Culver *et al.*, 1991). The *N* gene has been cloned and the nucleotide sequence has been determined (Whitham *et al.*, 1994). The encoded, putative protein has sequence similarity to proteins encoded by genes for resistance to bacteria and fungi (Mindrinos *et al.*, 1994; Whitham *et al.*, 1994; Staskawicz *et al.*, 1995). The HR induced by bacteria, fungi and viruses also activates a non-specific inhibition termed systemic acquired resistance (SAR), which responds to various pathogenic agents (reviewed by Kessmann *et al.*,

1994; Ryals *et al.*, 1994). The TMV coat protein gene is the elicitor of such responses induced through the *N'* gene (Culver and Dawson, 1989) and it is believed that the TMV polymerase gene is the elicitor of the HR induced through the *N* gene (Padgett and Beachy, 1993). Both the HR and the SAR have been analyzed extensively at the molecular level, and both the nature of various pathogenesis-related proteins and SAR-inducing signals have been discerned (reviewed by Enyedi *et al.*, 1992; Dixon *et al.*, 1994; Lamb, 1994; Dangl *et al.*, 1995; Ryals *et al.*, 1995). Nevertheless, it is still unclear how the SAR or the HR prevent further virus infection. Some viruses are not confined by the HR (Matthews, 1991). Moreover, other viruses that induce an HR exist outside the lesions formed by the HR, but no longer continue the infection process, even though these particles are still infectious after extraction (Matthews, 1991). Clearly, there are additional plant responses involved in the localization of the virus besides the necrosis induced during the HR (de Jager and Wesseling, 1981). For example, the *N* gene-induced HR to TMV can be inhibited without affecting the localization of the virus (Takusari and Takahashi, 1979). Thus, the existence of such a viral inhibition response (IR) leading to localization may be linked or not linked to the HR. The work described below shows the presence of an IR, distinct from the HR in cowpea (*Vigna unguiculata*), to infection by cucumber mosaic virus (CMV).

CMV has an unusually large host range, encompassing ~1000 species of plants in 365 genera of 85 families (reviewed by Palukaitis *et al.*, 1992). While most isolates of CMV induce only an HR on the inoculated leaves of cowpea plants and do not infect either peas or beans, some isolates ('strains') infect all three leguminous hosts systemically (Whipple and Walker, 1941; Fulton, 1950; Edwards *et al.*, 1983; Davis and Hampton, 1986; Daniels and Campbell, 1992). Based on the level of nucleotide sequence similarity, isolates of CMV have been classified into two subgroups (Palukaitis *et al.*, 1992). Strains that can infect legumes only occur in subgroup I (Daniels and Campbell, 1992). The genome of CMV contains five genes located on three genomic RNAs, designated RNAs 1, 2 and 3, and expressed from either the genomic RNAs or two subgenomic RNAs (Peden and Symons, 1973; Schwinghamer and Symons, 1977; Palukaitis *et al.*, 1992; Ding *et al.*, 1994). When the genome of a resistance-breaking (B-; bean) strain of CMV was reassorted with the genome of a restricted (LS-; lettuce) strain of CMV, RNA 2 of B-CMV was shown to condition for infection in three leguminous species (Edwards *et al.*, 1983). RNA 2 encodes the 5'-proximal 98 kDa 2a protein, a component of the viral 'replicase' (Hayes and Buck, 1990), as well as the 3'-proximal 11 kDa 2b protein, involved in host-specific virus accumulation (Ding *et al.*, 1994).

Using full-length cDNA clones of each genomic CMV

Fig. 1. Systemic symptoms of CMV on cowpea plants, at 2 weeks p.i. Cowpea plants were inoculated with either CMV isolated from tobacco (Fny, FBF, F/AT) or CMV RNAs transcribed from cDNA clones (F/A, F/T); CONT, non-inoculated control plants. (Limited necrosis on leaves inoculated with F/T-CMV RNAs is not due to the effects of virus, but due to mechanical damage.) Fny, Fny-CMV (wt); FBF is the pseudorecombinant containing Fny-CMV RNAs 1 and 3, and B-CMV RNA 2; F/A and F/T are Fny-CMV RNAs containing mutations in RNA 2 at nucleotide positions 1978 or 2007, respectively; F/AT is the double mutant with changes at both RNA 2 nucleotide positions 1978 and 2007.

RNA, from which infectious RNA transcripts can be generated, we have mapped the specific sequences involved in resistance-breakage in cowpea, and have demonstrated that there are two host responses occurring during infection of cowpea: an IR and an HR, which are induced by a different combination of specific sequence alterations. We also show that the IR affects viral RNA synthesis in isolated cells and present a model for the activation of the IR leading to virus localization.

Results

Induction of HR in cowpea maps to a specific domain encoding only three amino acid changes

Since B-CMV did not induce an HR in cowpea (Edwards *et al.*, 1983), but the Fny-strain of CMV did (P. Palukaitis, unpublished results), we cloned a cDNA copy of RNA 2 of B-CMV, pBCMV2, behind a T7 RNA polymerase promoter. RNA transcribed from pBCMV2 was combined with RNAs 1 and 3 of Fny-CMV [also transcribed from full-length cDNA clones (Rizzo and Palukaitis, 1990)], to generate the reassorted genome designated FBF (for Fny-CMV RNA 1, B-CMV RNA 2, and Fny-CMV RNA 3). The reassorted RNAs were inoculated either to the systemic host tobacco, or directly to cowpea. FBF-CMV purified from the tobacco plants was also inoculated to cowpea plants. In both cases, the cowpea plants did

not show an HR, but rather showed typical light-green/dark-green systemic mosaic symptoms (Figure 1). Accumulation of FBF-CMV RNA could be detected in symptom-bearing, non-inoculated leaves (not shown). Fny-CMV constructed from transcripts of the full-length cDNA clones pFny109, pFny209, and pFny309 (Rizzo and Palukaitis, 1990), only produced a necrotic local response (or lesion), 24–36 h post-inoculation (p.i.) (not shown) and no systemic infection ensued (Figure 1), as was reported in earlier studies using other CMV strains (Whipple and Walker, 1941; Gonda and Symons, 1979; Hanada and Tochiara, 1980; Edwards *et al.*, 1983; Daniels and Campbell, 1992). No viral RNAs accumulated in the upper leaves of cowpea plants inoculated by Fny-CMV and little-to-no accumulated viral RNAs could be detected in the inoculated leaves (data not shown).

After confirming that the determinants for systemic mosaic versus an HR mapped to RNA 2, we constructed a series of chimeric viruses using pBCMV2 and pFny209 (Figure 2) to further delimit the viral sequences involved in the induction of an HR. The chimeric RNA 2 transcripts were inoculated to tobacco plants along with Fny-CMV RNAs 1 and 3. In all cases, the chimeras showed typical CMV symptoms on tobacco 4–5 days p.i., and the yield of virus was similar for the various chimeras (data not presented), indicating that the viruses were fully functional in tobacco. Chimeric viruses purified from tobacco were

Fig. 2. Localization of sequences in CMV RNA 2 specifying the type of pathogenicity on cowpea. The nature of the RNA 2 used in the inocula, as well as the contribution from the Fny-CMV 2 parent (open rectangle) or the B-CMV RNA 2 parent (solid rectangle) are shown, along with the pathogenicity induced by virus containing the RNA 2 specified. The RNA 2 chimeras are named after the progenitor parental sequences (F or B), as well as the internal restriction endonuclease sites (shown above the Fny-CMV RNA 2) used to construct the chimeras (X, N, and/or E). The rectangles indicate the 2a gene, while the flanking lines indicate the 5' and 3' non-translated regions.

inoculated to cowpea plants. In cowpea, the data showed that sequences of B-CMV RNA 2 specifying systemic mosaic versus those of Fny-CMV RNA 2 inducing the HR are localized within the same ~280 nt region between the *Nco*I and *Eco*R1 restriction endonuclease sites (Figure 2); i.e. the RNA 2 chimera BFB/NE gave an HR, even though it contains all but 280 nt derived from B-CMV RNA 2, and the reciprocal RNA 2 chimera, FBF/NE, showed the opposite phenotype. Since the events occurring during the HR limited the virus to the site of inoculation (Figure 2), this region of the genome is associated with both the HR and the localization response by cowpea plants.

The nucleotide sequences between the *Nco*I and *Eco*R1 sites of pBCMV2 were determined, and showed 10 nt differences from pFny209 in this region (data not presented). The level of sequence diversity seen here (10 out of 280 nt; ~3%) is typical for variation among CMV strains in the same taxonomic subgroup (Palukaitis *et al.*, 1992). Seven of the changes were silent and three altered the putative sequence of the encoded 2a protein (Table I). To further delimit which sequences might be involved in the induction of the HR, we compared the sequences in this region of other subgroup I CMV strains for which the phenotype (local lesion induction) on cowpea and the nucleotide sequences were known (strains Y, O and K), with those of legume-infecting strains of CMV we determined here (strains F100, F415C, Pg, VE111, VE97, UH, and T136). Strain 29D, the partial nucleotide sequence of which was also determined here, induced an HR on cowpea and not a systemic mosaic. The complete nucleotide sequence of a legume-infecting CMV strain from Japan (LeJ) was obtained from the GenBank library. In all, the sequence comparisons showed a correlation of

Table I. Selective nucleotide and amino acid sequence comparison among CMV strains

CMV strain ^a	Nucleotide (amino acid) at position number		
	1936 (617)	1978 (631)	2007 (641)
Fny	CUG (Leu)	UUC (Phe)	GCC (Ala)
Y	CUG (Leu)	UUC (Phe)	GCA (Ala)
O	CUG (Leu)	UUC (Phe)	GCA (Ala)
K	GUA (Val)	UUC (Phe)	GCA (Ala)
29D	CUG (Leu)	UUC (Phe)	GCC (Ala)
B	CAG (Gln)	UAC (Tyr)	UCA (Ser)
F100	CAG (Gln)	UAC (Tyr)	UCA (Ser)
F415C	CAG (Gln)	UAC (Tyr)	UCA (Ser)
Pg	CAG (Gln)	UAC (Tyr)	UCA (Ser)
VE111	GCG (Ala)	UAC (Tyr)	UCU (Ser)
VE97	AUG (Met)	UAC (Tyr)	UCA (Ser)
UH	CAG (Gln)	UAC (Tyr)	UCA (Ser)
T136	CAG (Gln)	UAC (Tyr)	UCA (Ser)
LeJ	CUG (Leu)	UAC (Tyr)	UCA (Ser)

^aStrains Fny, Y, O, K and 29D all give local lesions on cowpea. The other strains infect cowpea systemically without giving a hypersensitive response.

conserved nucleotide sequences with the HR phenotype at only two of the three identified positions, nucleotides 1978 and 2007 (Table I), in the codons for amino acids 631 and 641, respectively, of the 2a protein. In the codon for amino acid 617 (at nucleotide 1936), there were different amino acid sequences encoded by the legume strains compared with those that induce an HR, in all cases except for strain LeJ. In addition, there was variation in amino acid sequence at this position in strains within the same phenotype class (Table I). There was, however, a strict conservation of sequence at amino acid positions 631 and 641 in strains within each phenotype class, although there was variation in the nucleotide sequence of the codon for amino acid 641.

Specific sequence combinations within CMV RNA 2 induce necrosis and restrict virus movement in cowpea

To ascertain whether one or both of the two conserved changes noted above determined the HR and localization phenotypes, the sequences at nucleotide positions 1978 and 2007 were altered by site-directed mutagenesis in pFny209 to correspond to those sequences present in pBCMV2. These mutants were designated F/A (position 1978), F/T (position 2007), and F/AT (both positions) (Figure 3C). Transcripts of each mutant cDNA were combined with Fny-CMV RNAs 1 and 3 and inoculated to either tobacco or cowpea plants. Like Fny-CMV, the F/T-CMV mutant passed through tobacco induced necrotic local lesions in cowpea; however, several weeks later, systemic mosaic symptoms were observed (not shown). After virus was purified and the mutated region of RNA 2 was analyzed by dideoxynucleotide-sequencing, several additional nucleotide changes were observed including one that altered nucleotide 1978, creating a mutant equivalent to F/AT (Figure 3C). When CMV RNA transcripts containing the F/T mutant were inoculated directly to cowpea, they induced necrotic local lesions (Figure 4) but did not infect the plants systemically (Figure 1). By contrast, inoculation of cowpeas with CMV RNAs

Fig. 3. Fny-CMV RNA 2 and 2a protein sequences as well as the sequences of mutants generated in pFny209 that determine the pathogenicity in cowpea. (A) Diagram showing the portion of RNA 2 encoding the 2a protein (open rectangle) flanked by the 5' and 3' non-translated regions. The domain between the *Nco*I and *Eco*RI sites responsible for pathogenicity on cowpea is indicated. (B) The amino acid sequence encoded by the region of the Fny-CMV 2a gene indicated in (A), with amino acid sequences that differ between Fny-CMV 2a and B-CMV 2a shown in bold, and the conserved GDD present in viral polymerases shown underlined. (C) The amino acid sequences of Fny-CMV 2a protein from positions 629 to 644, encoded by nucleotides 1971 to 2018 are shown, as well as the symptoms induced in cowpea by Fny-CMV, along with the nucleotide and amino acid sequence differences in B-CMV RNA 2 and the mutant cDNA clones F/A, F/T and F/AT, plus their corresponding pathogenicities.

containing mutant F/AT did not induce an HR, but induced mild, chlorotic lesions on the inoculated leaf (Figure 4) followed by systemic chlorosis (Figure 1). Thus, the nucleotide change at position 2007 was not sufficient to alter the HR phenotype induced by Fny-CMV RNA 2, but required, in addition, a change at nucleotide 1978. F/T-CMV did not appear to have any selective advantage in tobacco over F/AT-CMV, and mutants in F/T-CMV generated in tobacco at position 1978 were then selected for after passage to cowpea.

Infection of cowpea by CMV RNAs including mutant F/A (with a change solely at nucleotide 1978) gave an unexpected phenotype. Instead of pinpoint necrotic lesions appearing 1 day p.i. and enlarging only slightly over the next few days—as for CMV RNAs containing the F/T mutant (Figure 4)—no lesions appeared with F/A-CMV 1 day p.i., but rather larger necrotic lesions appeared 2 days p.i. (Figure 4). The necrotic reaction continued to spread both throughout the inoculated leaf on subsequent days (Figure 4) as well as systemically, until the entire plant collapsed (Figure 1). Analysis of the systemic leaves before the onset of systemic necrosis showed the presence of viral RNA in the systemic tissue (data not presented). Partial sequence analysis of RNA 2 of this viral RNA confirmed the absence of additional mutations between the *Nco*I and *Eco*RI restriction sites (data not shown). Thus, both positions 1978 and 2007 of Fny-CMV RNA 2 are involved in the induction of the HR, while position 1978 is also involved in inducing the IR leading to virus localization. In addition, necrosis due to the HR does not in itself lead to the IR.

CMV RNA accumulation is inhibited in cowpea protoplasts

Cowpea protoplast were inoculated with Fny-CMV RNAs as well as Fny-CMV RNAs containing mutations in RNA

2 at nucleotide positions 1978 and 2007 (the F/AT mutant, referred to as F/AT-CMV RNAs). The accumulation kinetics of viral RNA and viral-encoded proteins were examined (Figure 5 and data not shown) to determine whether there were differences in the rates of accumulation of viral RNAs or the viral-encoded proteins, that could account for the difference in the observed responses by cowpea plants to the mutants. Although cowpea protoplasts supported the replication of both the Fny-CMV and F/AT-CMV RNAs (Figure 5A) and showed the same initial kinetics of RNA accumulation, the Fny-CMV RNAs did not accumulate to as high a level at 24 and 48 h p.i. as did the resistance-breaking F/AT-CMV RNAs (Figure 5A and B). In addition, there was an apparent drop in the level of accumulated genomic RNAs at 12 h p.i. for Fny-CMV, but not for the F/AT-CMV mutant. This was observed in several, independent experiments and also could be seen in the time course plot shown in Figure 5B. In some experiments, a plateau rather than a slight drop in the level of Fny-CMV RNA accumulation was observed, and in other experiments, the drop or plateau was detectable at the 15 h time point rather than the 12 h time point (see also Figure 5D). However, in all of the experiments, the levels of accumulation of Fny-CMV RNAs in cowpea protoplasts at 24 and 48 h p.i. were always lower than from protoplasts inoculated with F/AT-CMV RNAs.

While it has been documented that cowpea protoplasts inoculated with CMV do not become necrotic (Koike *et al.*, 1977; Gonda and Symons, 1979; Nasu *et al.*, 1996), the data shown here indicate that there is a host response that may either inhibit replication of the viral RNAs, or accelerate the turnover of the viral RNAs during a period prior to the onset of the HR pathway in whole plants. Analysis of the viral-encoded proteins confirmed that this host IR affected the accumulation kinetics of the 2a protein

Fig. 4. Symptoms induced on the inoculated leaves of cowpea plants at 1–4 days after inoculation (dpi) with CMV RNAs containing the RNA 2 mutants F/A, F/T or F/AT. A non-inoculated control (CONT) leaf is also shown. Arrowheads indicate pin-point necrotic lesions in F/T as well as larger necrotic lesions in F/A and mild, chlorotic lesions in F/AT. The larger necrotic lesions in F/A coalesced and led to complete collapse of the tissue at 3 or 4 days p.i.

(Figure 5C), which together with the 1a protein and host proteins constitute the viral replicase (Hayes and Buck, 1990). The 2a protein reached a peak of accumulation early for Fny-CMV, followed by a reduction in the level of 2a protein that only increased slightly over the remaining time course (Figure 5C). The 2a protein of F/AT-CMV showed continued accumulation from 3 to 24 h p.i. (Figure 5C), as was previously observed for Fny-CMV in squash protoplasts (Gal-On *et al.*, 1994, 1995). The 1a protein has never been detected in protoplasts and is detected only transiently in whole plants (Gal-On *et al.*, 1994, 1995). The 3a movement protein and the coat protein did not show any difference in accumulation kinetics over the time course examined (not shown; see also Figure 3 of Nasu *et al.*, 1996). Thus, the two mutations at positions 1978 and 2007, which alter the amino acid sequence of the 2a protein at positions 631 and 641 respectively, may also affect the stability of either the 2a protein, or the RNA (2) encoding the 2a protein.

Attempts to determine whether viral RNA biosynthesis actually ceased 12–15 h p.i., or whether viral RNA

degradation was increased, were unsuccessful. The ^{32}P label incorporated into viral RNAs in 3-h pulses was masked by the incorporation of ^{32}P into the host cytoplasmic and chloroplast rRNAs. Attempts to isolate virus particles containing pulse-labeled viral RNAs were also unsuccessful, even when non-radioactive carrier virions were added to the lysed protoplasts (data not shown).

Since infection by the F/A-CMV mutant (a change at position 1978 alone) resulted in the induction of an HR, but not a localization of infection, if the IR in protoplasts is linked to the localization of infection in plants, then the F/A-CMV mutant should also exhibit increased RNA accumulation kinetics vis-à-vis Fny-CMV; however, if the IR in protoplasts is linked to the HR, then the F/A-CMV mutant should exhibit RNA accumulation kinetics similar to that of Fny-CMV. Protoplasts infected with the F/A-CMV RNAs showed an increased RNA accumulation profile relative to Fny-CMV, similar to that of the F/AT-CMV mutant (Figure 5D versus Figure 5A). This demonstrates that the IR and the HR could be uncoupled (Figure 5A versus Figure 5D) just as the HR and the

Fig. 5. Kinetics of accumulation in cowpea protoplasts. Cowpea protoplasts were infected with either Fny-CMV RNAs (A–D), F/AT-CMV RNAs (A–C), or F/A-CMV RNAs (D) and aliquots were taken at the times (in hours) indicated for analysis of either CMV RNA levels by Northern blot hybridization (A and D), or 2a protein levels by Western blotting (C). The radioactivity corresponding to the four CMV RNAs in the blot in (A) was quantified and plotted in (B). The positions of genomic CMV RNAs 1, 2 and 3 and subgenomic RNA 4 are indicated in (A) and (D), as is the position of the 2a protein in (C).

localization of infection could be uncoupled (Figures 1 and 4).

Discussion

The ability of leguminous strains of CMV to break the resistance to CMV and infect cowpea plants systemically without the induction of an HR mapped to two nucleotide positions in the 2a gene. It would seem more probable that this phenotype is controlled by the changes at the two amino acids (631 and 641) in the 2a protein encoded by the nucleotide sequences including position 1978 and 2007 (Table 1 and Figure 3C), rather than due to an effect of the RNA sequence itself, although this cannot be demonstrated conclusively with the current data. The

replacement of Phe and Ala by Tyr and Ser, respectively, does not appear to involve radical changes in sequence; however, both Tyr and Ser contain hydroxyl groups, which might either change the hydrophobicity of the local environment, or be subjected to phosphorylation by either mitogen-activated protein kinases (MAPKs) or other plant kinases, that could alter their interactions with other cellular components. These two amino acids are located close to the GDD motif (amino acids 609–611) which is conserved in viral polymerases (Figure 3B) and is accessible to interactions with other proteins (e.g. antibodies) as a component of the viral replicase (Hayes *et al.*, 1994). There are no data indicating that amino acids 631 and 641 are involved in the replication process *per se*, and the levels of the mutant viruses were similar to Fny-CMV in tobacco (data not presented). However, the changes in sequence do affect the accumulation of viral RNAs in cowpea protoplasts (Figure 5).

A UV-induced mutant of alfalfa mosaic virus RNA 2, which also failed to induce an HR in cowpeas, was found to accumulate to higher levels in cowpea protoplasts (Roosien *et al.*, 1983). Perhaps similar mechanisms of IR and HR are involved, since some strains of alfalfa mosaic virus induced an HR and remained localized, while other strains induced an HR but still gave a systemic infection.

HR-inducing strains of CMV do not induce an HR in protoplasts (Koike *et al.*, 1977; Gonda and Symons, 1979; Nasu *et al.*, 1996). This is not only because of a possible need for cell wall-associated components that are absent from protoplasts (Dixon, *et al.*, 1994), since movement-defective mutants of CMV are unable to induce a visible HR in cowpea plants (Suzuki *et al.*, 1991). On the other hand, a coat protein deletion mutant which demonstrated limited cell-to-cell movement in tobacco could still induce an HR in cowpea (Suzuki *et al.*, 1991). At the same time, it is not clear to what extent cell-to-cell movement and significant virus replication would have occurred beyond several levels of adjacent cells by 24 h p.i., when the infected cells collapsed. The size of the lesion (~0.5 mm) suggests that it contains 50–100 cells (Mise and Ahlquist, 1995).

In an earlier study on the kinetics of Q-CMV infection in cowpea protoplasts, where only an HR-inducing strain was available, Gonda and Symons (1979) observed that accumulation of coat protein continued for 50 h p.i.; however, in a pulse-labeling study, they observed that the rate of coat protein synthesis dropped severely after 15 h p.i. We suggest that they also were observing a manifestation of the IR, similar to that observed here for Fny-CMV.

The biochemical events occurring during the formation of the HR against CMV in cowpea have been described (Kato and Misawa, 1976). The addition of free radical scavengers and an inhibitor of lipoxygenase retarded lesion development, indicating that oxidation of polyunsaturated lipids and the generation of free radicals and hydroperoxides are involved in the HR. Changes in the above constituents occurred 5–8 h p.i., before the time that the effects of the IR on the 2a protein accumulation (Figure 5C) and on viral RNA accumulation (Figure 5A and D) became manifested in protoplasts, and well before the time that the HR was observed in leaves (18–24 h p.i.). The timing of events suggests that the HR must take place soon after virus replication is initiated, and may occur by

an 'oxidative burst' (Dixon *et al.*, 1994) rather than by apoptosis.

The ability of the two resistance-breaking mutants, F/AT-CMV and F/A-CMV, to accumulate to higher levels in protoplasts (Figure 5A and D) than Fny-CMV suggests that the transient IR may be part of the localization of infection (Figure 4), since the Fny/A-CMV mutant still induced an HR but neither an IR nor localization (Figures 1 and 4 compared with Figure 5). Based on the data obtained, it is not possible to distinguish unequivocally between the reduction in the level of accumulating 2a protein being due to a decrease in the level of RNA 2, which encodes the 2a protein, or the reduced accumulation of RNA 2 resulting from a decrease in the level of the 2a polymerase protein, or both events. However, we propose that the 2a protein may be the target of the IR, possibly involving the inability to phosphorylate amino acid 631 in Fny-CMV and F/T-CMV. This may result in a slight effect on the accumulation of the viral RNA, which then begins a feedback cycle that is manifested as a plateau or decrease in accumulation of viral RNA and 2a protein over the next few hours. This model predicts that phosphorylation could result in the activation of a host response that prevents the HR. Amino acids 629–631 in the 2a protein encoded by FBF-, F/A-, and F/AT-CMV are Thr-Leu-Tyr (Figure 3C). The consensus sequence for the phosphorylation site of MAPKs involved in plant signal transduction is Thr-X-Tyr, in which both the Thr and the Tyr residues must be phosphorylated for activation of the MAPKs (Hirt, 1997).

A decrease in the accumulation of the 2a protein could also affect the movement of CMV, if the 2a protein has a role in virus movement. Such a role is suggested by two observations. First, replicase-mediated resistance to CMV, engendered by expression of a defective 2a gene, inhibited both virus replication and virus movement; these two inhibitory effects could be overcome separately using RNA 2 chimeras of Fny-CMV and a resistance-breaking strain, K-CMV (Hellwald and Palukaitis, 1995). Second, the RNA accumulation kinetics for Fny-CMV and either FBF-CMV or F/AT-CMV are identical in pea protoplasts, while F/AT-CMV and FBF-CMV can move in pea plants, but Fny-CMV cannot (C.-H.Kim and P.Palukaitis, unpublished data). Thus, if movement mediated by the 2a protein is retarded by the IR, then the HR, potentially associated with the inability to phosphorylate either or both amino acids at positions 631 and 641, may result in the localization of the virus to the lesion. Alternatively, the slight IR observed may be sufficient to cause a delay in the infection process long enough for some other host response to be activated.

A naturally occurring mutant of cowpea (cv. Black) was described in which the plants no longer responded with an HR but rather showed a 'compatible interaction' in which the virus infected the plant systemically, without inducing necrosis. The HR was dominant over the compatible response, and appeared to involve a single gene (Sinclair and Walker, 1955). More recently, a cowpea line was described in which CMV was no longer localized, but the HR was still induced, resulting in a systemic necrosis (Nasu *et al.*, 1996). This localization response was also shown to be due to a single, dominant gene. In addition, the description by those authors of systemic

mosaic occurring when larger plants of the susceptible line were inoculated, parallels our own experience of limited necrosis when larger cowpea plants of our cultivar were inoculated with the F/A-CMV mutant (data not shown). Thus, it appears that the *Cry* gene described by Nasu *et al.* (1996) controls the IR rather than the HR, while the *Cm* gene(s) of Sinclair and Walker (1955) may have controlled both responses.

The IR to CMV in cowpea is transient, and if it inhibits CMV RNA synthesis, it does so for only a few hours. Further work will be required to determine whether the IR in cowpea actually causes CMV replication to cease and whether, once activated, the IR in cowpea has any effect on the RNA accumulation of other viruses that do not induce the HR and IR in cowpea. Such a non-specific response has been observed for the IR in potato containing the *Rx* gene (Köhm *et al.*, 1993). In potato, an extreme resistance to potato virus X is specified by the *Rx* gene and is elicited by the viral coat protein (Goulden *et al.*, 1993). This also is manifested in protoplasts, where activation of the IR prevents viral RNA accumulation very early post-inoculation and is maintained throughout the time course (Köhm *et al.*, 1993). Whether this is a general or rare response of resistance genes affecting replication remains to be established.

Materials and methods

Plants, virus inoculation, RNA extraction and analysis

Nicotiana tabacum cv. Turkish Samsun NN (tobacco) plants were inoculated at the four- to six-leaf stage, as previously described (Rizzo and Palukaitis, 1990). The inocula were either non-recombinant viruses, or RNA transcripts generated from cDNA clones. The cDNA clones were from either Fny-CMV RNAs 1–3 (Rizzo and Palukaitis, 1990), or Fny-CMV RNAs 1 plus 3, and either B-CMV RNA 2, or various chimeras formed between cDNA clones of Fny-CMV RNA 2 (pFny 209) and B-CMV RNA 2 (pBCMV2). Viruses were purified from infected tobacco plants and viral RNAs were extracted and recovered as described by Palukaitis *et al.* (1992) and Kaplan *et al.* (1995), respectively. Either purified viruses or viral RNA transcripts were inoculated to primary leaves of *Vigna unguiculata* (cowpea) plants which were maintained in environmentally controlled growth chambers at 25–28°C with a 14:10 h light:dark cycle. Total nucleic acids were extracted from infected cowpea leaves as described for tobacco leaves by Gal-On *et al.* (1995). Viral RNAs purified from systemically infected cowpea plants were sequenced by dideoxynucleotide chain termination using a kit supplied by US Biochemicals (now Amersham) and [α -³²P]dATP (Amersham). An oligonucleotide complementary to Fny-CMV RNA 2 sequences 2043–2060 was used as a primer for sequencing the specific region described, in RNA 2 of the various CMV strains. Strains F100, F415C, VE111, VE97, T136 and 29D were obtained from Dr V.Lisa (C.N.R., Torino, Italy). Strains Pg and UH were obtained from the late Dr R.I.B.Francki (University of Adelaide, Australia).

Preparation, electroporation, extraction and analysis of cowpea protoplasts

Mesophyll protoplasts were prepared from fully expanded primary leaves of cowpea plants harvested 8–10 days after planting. The plants were grown as described above in environmentally controlled chambers. The detached leaves were surface-sterilized for 10 min in 10% hypochlorite and were rinsed extensively with distilled water. The lower epidermis was removed from each leaf and the leaves were floated for 10 min abaxial side down, on 0.45 M mannitol containing 0.5% (w/v) polyvinylpyrrolidone. The leaves were then incubated for 3 h at 30°C in 0.45 M mannitol containing 0.5% (w/v) macerozyme, 1.5% (w/v) cellulase (both Onozuku B-10, from Yakult Pharmaceutical Co., Tokyo, Japan), with the pH adjusted to 5.6 using saturated succinic acid; 15 ml/leaf/Petri dish. The leaf skeletons were removed and the protoplasts pooled, filtered through pre-wetted Miracloth, and collected by centrifugation at 100 g for 3 min. The protoplasts were resuspended in 15%

(w/v) sucrose, overlaid with 0.45 M mannitol and centrifuged as above. The floating protoplasts were washed three times with 0.45 M mannitol and diluted to 0.5×10^6 protoplasts/ml. The protoplasts were inoculated by electroporation as previously described (Gal-On *et al.*, 1994), except that the RNA inoculum concentration was 5 µg/ml, and two pulses of 300 V for 5 ms each were used. The protoplasts were then washed, incubated for the times indicated, extracted and analyzed, all as described for squash protoplasts by Gal-On *et al.* (1994), except that the mannitol concentration in the incubation and washing media was 0.45 M. RNAs were analyzed by Northern blot hybridization using a ^{32} P-labeled, RNA probe complementary to the 3' end 200 nt common to the three genomic CMV RNAs (Gal-On *et al.*, 1994). Proteins were analyzed by immunoprobable electrophoretograms with antisera specific to the 1a, 2a, 3a and coat proteins of CMV (Gal-On *et al.*, 1994, 1995; Kaplan *et al.*, 1995). RNAs were quantified from original nitrocellulose membrane by liquid scintillation counting (Gal-On *et al.*, 1994).

Generation of pBCM2, chimeric cDNA constructs and mutants

Full-length cDNA to B-CMV RNA 2 was generated using reverse transcription and the polymerase chain reaction (RT-PCR), to produce two half-length cDNA molecules that were than 'joined' by strand-overlap PCR (Sambrook *et al.*, 1989). Primers complementary to the 3' terminal region and both identical to the 5' terminal region and containing a T7 RNA polymerase promoter were described previously (Hellwald and Palukaitis, 1994), for the generation of a full-length cDNA to K-CMV RNA 2. The two internal primers were complementary to and identical to nucleotides 1533–1549 of the Fny-CMV RNA 2 sequences (Rizzo and Palukaitis, 1988).

Chimeras were made between pFny209 and pBCM2, using the common, unique restriction endonuclease sites *Bam*H1, *Xho*I, *Nco*I, *Eco*RI and *Pst*I, and standard procedures described by Sambrook *et al.* (1989). The identities of the chimeras were confirmed by partial dideoxynucleotide chain-termination sequencing of the plasmid DNA using primers specific to CMV RNA 2 (Hellwald and Palukaitis, 1994, 1995).

Site-directed mutagenesis was performed to alter either nucleotide 1978, nucleotide 2007, or both, by the two-step PCR procedure described by Higuchi *et al.* (1988). The complementary paired primers covering and flanking the mutation sites were 5'-ACAACCTTTACAACATGGAA-3' (plus strand) and 5'-TTCCATGTTGTAAAGAGTTGT-3' (minus strand) for nucleotide 1978 (underlined); 5'-GATGGAACCTTCCGTACCATAT-3' (plus strand) and 5'-ATATGGTACGGAAGGTTCCATC-3' (minus strand) for nucleotide 2007 (underlined); and both sets sequentially, to create the double mutant F/AT (1978 and 2007). The reaction conditions, terminal primers and characterization of the mutations were all done as described by Hellwald and Palukaitis (1995).

Acknowledgements

This research was supported by Grant No. 92-37303-7799 from the US Department of Agriculture National Research Initiative Competitive Grants Program.

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Received on December 13, 1996; revised on March 14, 1997