tures analyzed is summarized in Table 1. We extracted the sulfur by mixing the fungi in chloroform for 10 minutes. We removed the fungal structures by filtration and concentrated the chloroform extract under vacuum at 30°C.

Two analytical methods were used to test for elemental sulfur. In the first method the chloroform extract was analyzed by thin-layer chromatography (TLC) according to the following procedure: 10 to 50 μl of the extract was deposited on a KHF_{254} TLC plate. The solvent system was chloroform. In some extracts natural fungal pigments were mixed with sulfur, and cyclohexane-chloroform (99 : 1) was used to ensure a better separation. In each case a sulfur standard in chloroform was deposited with the fungal extract and was cochromatographed under the same conditions. The R_f of free sulfur varied between 0.85 and 0.90. Triphenyltetrazolium chloride (4 percent in methanol) mixed with an equal amount of sodium hydroxide solution (4 percent in methanol) was sprayed on the plates and developed for 10 minutes at 110°C. Sulfur, in the S_8 form, develops a red spot in the presence of this reagent.

The second method of analysis was based on the reaction of free sulfur with cysteine to produce H_2S (*8*). The chloroform extract was evaporated under vacuum to dryness, and the residue was dissolved in 1 ml of hot absolute ethanol. Then 1 ml of a cysteine solution (0.25*N*, *p*H 6.8) was added to the ethanol extract. The mixture was placed in a sulfide test tube (Fig. 1). This apparatus was placed in a thermostatically controlled water bath and gently shaken at 37°C for 1 hour. Then two drops of concentrated H_2SO_4 were added. The temperature of the bath was increased to 55°C and maintained at this temperature for ½ hour. The test was considered positive if the sulfide-sensitive paper became black. The reaction was more or less intense depending on the quantity of free sulfur present. In cases where both the TLC analysis and the sulfide test were positive, we concluded that the fungal structures contained free sulfur.

The presence of elemental sulfur in a great number of fungi, from Myxomycetes to Basidiomycetes (Table 1), especially in the self-inhibited and dormant structures, is important because these dormant fungal organs (spores, chlamydospores, sclerotia) characteristically have a reduced respiratory capacity (*5*). Miller *et al.* (*9*) and Tweedy and Turner (*10*) determined that, when free sulfur is added to fungal spores, it acts as a hydrogen acceptor in hydrogenation and dehydrogenation reactions, particularly in the terminal respiratory chain between cytochrome b and cytochrome c, with a concomitant production of H_2S. This production of H_2S could account for the low respiratory capacity. For example, Schmit and Brody have demonstrated that conidia of *Neurospora crassa* are dormant spores with a low metabolic rate and an endogenous respiratory capacity lower than that of mycelia (*11*). We found that these conidia contain free sulfur (Table 1). Elemental sulfur has never been detected in vegetative hyphae of *Phomopsis viticola*; it appears at the stage where pycnidia differentiate. Therefore, a relationship seems to exist between dormancy and the presence of elemental sulfur.

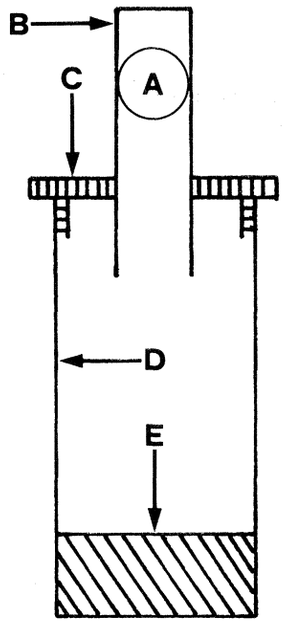

Fig. 1. Hydrogen sulfide test tube: sulfide-sensitive paper (*A*), small glass tube (*B*), polyethylene cap (*C*), large glass tube (*D*) containing the solution to be analyzed (*E*).

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Cucumber Mosaic Virus Associated RNA 5: Causal Agent for Tomato Necrosis

Abstract. *A small replicating RNA, encapsidated with and dependent on, but not part of the viral genome, modifies disease expression depending on the host. In tomato plants, it causes a lethal necrotic disease which is probably the same as that which, in 1972, destroyed most of the field tomato crop in large regions of the French Alsace.*

In 1972, field tomato plants in large regions of the French Alsace were stricken with a severe necrotic disorder of epidemic proportions. As a result, almost the entire field tomato harvest in that part of France was annihilated (*1*). Prior to 1972, this "tomato necrosis" disorder had only been observed sporadically, as limited outbreaks, in different parts of France (*2*). In 1974, conclusive evidence was given that the disorder was of viral etiology and somehow associated with cucumber mosaic virus (CMV) infection. Several well-characterized cloned strains of CMV were shown capable of inducing tomato necrosis in greenhouse experiments (*3*). However, because the more characteristic CMV symptoms of chlorosis and "fern leaf" syndrome (*4*) were also observed in these experiments, and because the incidence of necrosis was variable, its precise relationship to CMV was unresolved. Several explanations were suggested to account for this variability: (i) variable growing conditions of the infected plants; (ii) the possibility of there still being mixtures of necrotic and non-necrotic CMV strains in the inoculums; (iii) instability of the inoculated CMV strains and their subsequent mutation; (iv) varying states of CMV's divided genome (*5*) in the inoculums or in the infected plants.

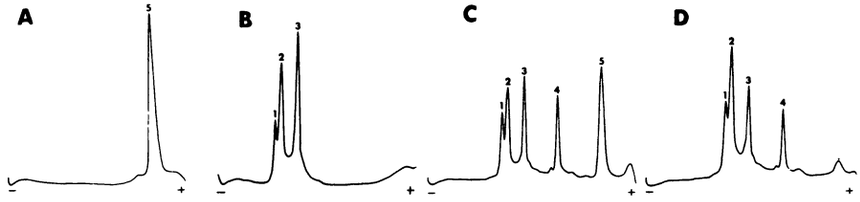

Fig. 1. Polyacrylamide gel electrophoresis patterns displaying RNA component composition of RNA preparations from CMV-S that were used to inoculate tomato plants, and of the RNA in virus isolated from infected tomato plants. (A) CARNA 5 preparation used in mixture with RNA 1+2+3 to inoculate group a plants. (B) RNA 1+2+3 used in mixture with CARNA 5 to inoculate group a plants and used to inoculate (by itself) group b plants. (C) RNA from CMV isolated from group a plants; CARNA 5 is present. (D) RNA from CMV isolated from group b plants. CARNA 5 is absent. Polyacrylamide electrophoresis on 2.4 percent gels was carried out as described. The last peak in panels C and D is viral protein (*6*).

One of the strains used in the above experiments (*3*) was the South African or S strain of CMV. The composition and molecular organization of this CMV strain has been studied extensively (*6*). In the course of that work we found that, in addition to the three genomic RNA's 1, 2, and 3 and RNA 4 [the nucleotide sequence of which also occurs in RNA 3 (*7*)], CMV-S virions contain a fifth RNA component of 100,000 daltons, which is not part of CMV's divided genome. Recently, we have found that this low-molecular-weight CMV-associated RNA 5 (CARNA 5) was encapsidated with one or more of CMV's genomic RNA's (*8*), and that in host plants like tobacco it was synthesized in large quantities, provided that the three genomic RNA's were also present in the inoculum (*9*). We observed that under these conditions virus yield and specific infectivity were considerably reduced, and we concluded that CARNA 5 was a helper-dependent, replicating, defective, or satellite RNA, capable of severely depressing CMV multiplication in tobacco (*9*).

We now describe experiments that reveal a direct cause-effect relationship between the presence of CARNA 5 in inoculums of the genomic RNA's of CMV-S and the subsequent incidence of severe necrosis in tomato plants, resembling the tomato necrosis disease syndrome in France (*1–3*).

CMV-S (obtained from Dr. M. H. V. Van Regenmortel, South Africa) was propagated in squash (*Cucurbita pepo* L. 'Caserta Bush') or in tobacco (*Nicotiana tabacum* L. 'Xanthi nc') as described (*9*). The CMV-S propagated in squash contained larger proportions of the genomic RNA's (particularly RNA 1), whereas virus from tobacco contains large proportions of CARNA 5 (*9*). Purified from these two host species by the method of Lot *et al.* (*10*), CMV served as the source of the genomic RNA's 1, 2, and 3 and CARNA 5, respectively. The mixture of RNA's 1, 2, and 3 (hereafter called RNA 1+2+3) was separated from CARNA 5 by rate zonal centrifugation on sucrose gradients of the unfractionated RNA's (*9*). After several centrifugation cycles, preparations of RNA 1+2+3 and CARNA 5 showed no contamination with each other in polyacrylamide electrophoresis gels (Fig. 1, A and B). Furthermore, RNA 1+2+3 inoculated in tobacco, after one passage, gave rise to CMV that did not contain (or contained only negligible quantities of) CARNA 5, whereas CARNA 5 preparations inoculated in tobacco produced no CMV or CARNA 5 in either free or encapsidated form (*9*).

Three groups (a, b, and c) of six tomato plants each (*Lycopersicon esculentum* Mill. 'Rutgers'), 7 to 10 cm tall, were inoculated with (group a) a mixture of CMV-RNA 1+2+3 (10 μg/ml) with CARNA 5 (2.5 μg/ml), (group b) CMV-RNA 1+2+3 (10 μg/ml), both in 0.03*M* Na_2HPO_4, and (group c) 0.03*M* Na_2HPO_4. Inoculated plants were kept in an air-conditioned greenhouse at approximately 22°C. After 20 to 24 days, group a plants (inoculated with the mixture of CMV-RNA 1+2+3 and CARNA 5) displayed symptoms, either as a large spreading necrotic area on one or more inoculated leaflets, or as necrosis of the midribs of the petiolules (Fig. 2B). Shortly thereafter, epinasty of systemically infected leaflets was observed, and within another 2 to 3 days the midrib and lateral veins of these leaflets became necrotic and the plants chlorotic. In some plants the systemic symptom was observed first. The necrosis advanced down the petiolules to the petioles and the stem until the entire top of the plant was affected, first with a wilted appearance followed by drying of the tissue until it was brittle, leaving a turgid stem for only a few centimeters above the soil line 25 to 30 days after inoculation (Fig. 2A). All plants in group a

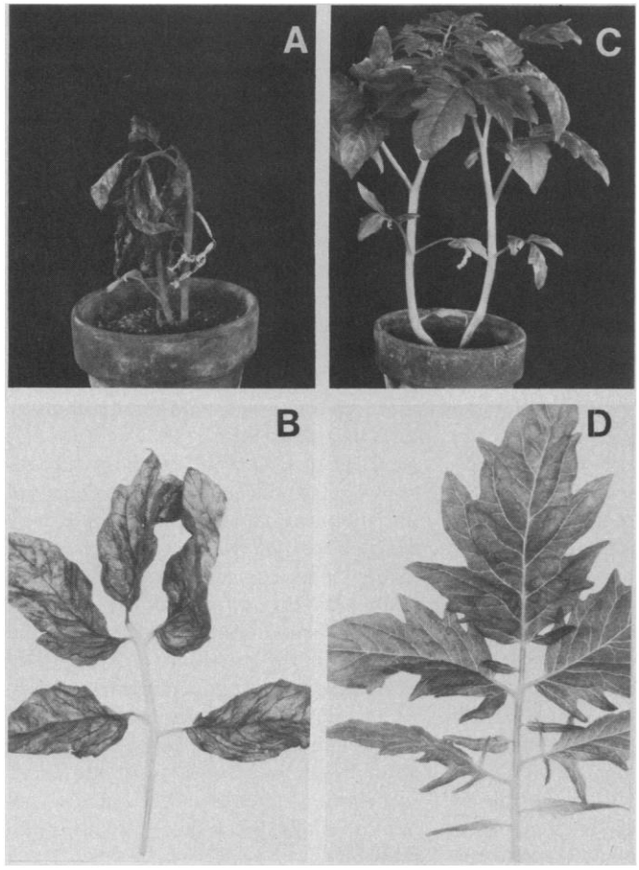

Fig. 2. Characteristic condition of tomato plants 28 days after inoculation with RNA components extracted and fractionated from preparations of CMV-S. (A and B) Two plants from group a, inoculated with a mixture of RNA 1+2+3 and CARNA 5. Both plants have collapsed (A). A leaf of one of these plants (B) displays clear symptoms of epinasty and vein necrosis, which has spread to petiolules and petiole. (C and D) Two plants from group b, inoculated with RNA 1+2+3 alone. The plants appear healthy (C). A leaf of one of these plants (D) shows mild mosaic and slightly narrowed leaflets.

were dead 4 to 6 weeks after inoculation. Group b plants (inoculated with CMV-RNA 1+2+3 alone) exhibited distinct mild mosaic after 22 to 28 days (Fig. 2C). During the following weeks, the leaflets became slightly rugose and tended to be narrower (Fig. 2D) than those on buffer-inoculated plants. Epinasty and necrosis have not been observed. Group b plants after 6 weeks were about 20 percent smaller than buffer-inoculated control plants in group c, which developed normally. The above experiment has been repeated three times during a 5-month period, each time with identical results. Two additional experiments were performed in a greenhouse without air-conditioning. The first, during May and June, gave similar results to the experiments described above. In the second, performed during June and July, when greenhouse temperatures exceeded 40°C most days, group a plants developed necrosis, but it was severely restricted to only a small number of leaves and petiolules. At later stages these plants developed normally. Group b plants developed only very weak, barely visible, chlorosis. This demonstrates that temperature conditions could have been an important factor in the variability of the incidence of CMV-induced tomato necrosis in France (*2, 3*).

To obtain further evidence that CARNA 5 had a role in the induction of tomato necrosis, virus was isolated (*10*) from group a plants, inoculated with a mixture of RNA 1+2+3 and CARNA 5, and with distinctly necrotic symptoms, and from group b plants, inoculated with RNA 1+2+3 alone, displaying systemic chlorosis. The RNA component composition of these preparations is shown in panels C and D of Fig. 1. Clearly, CARNA 5 is produced in large quantities in the necrotic tomato plants that were inoculated with RNA 1+2+3 plus CARNA 5, but not in those infected with only RNA 1+2+3.

We are now beginning to test less well characterized CMV isolates for the presence of, or their ability to help replicate CARNA 5. We have already found that CARNA 5 of different CMV strains can be freely exchanged among their helpers. We also succeeded in inducing strains that either carried no CARNA 5, or so little of it that they were not necrotic in tomato plants, to produce CARNA 5 upon admixture of the latter, and incite severe necrosis in tomato plants (*11*).

Our discovery of a small replicating RNA, which becomes encapsidated in the protein coat of its helper virus, and which, depending on the host plant, is able to modulate the pathogenicity of its helper or express a new pathogenicity of its own (or both), carries a number of important implications. It may offer the key to numerous enigmatic observations made in the past by plant pathologists in CMV-related disease outbreaks. (i) As we have shown, outbreaks of abnormally severe disease symptoms, like those in the Alsatian tomato necrosis, could be explained by the invasion of a CMV strain that carries CARNA 5, or by the introduction of CARNA 5 into plants endemically infected with a "mild" CMV strain, but capable of assisting the replication of CARNA 5. (ii) Conversely, our experiments in tobacco plants (*9, 11*) have demonstrated the essentially self-limiting nature of CARNA 5 replication. This property is characteristic for defective interfering systems (*12*) as well as for satellite viruses (*13*). It is probably also responsible for the recurrent character of the disease syndrome in many host plants chronically infected with CMV, where disease symptoms alternate with periods of partial remission (*14*). As predicted for defective interfering systems (*12*), in CMV infections these cyclical phenomena may be correlated with alternating levels of viral RNA and CARNA 5 in the infected tissues. (iii) The previously often observed weakening or "loss" of CMV isolates during repeated passages in certain hosts could also be explained by a "take-over" by CARNA 5, since during its replication the synthesis of the viral RNA's seems decreased (*9, 11*).

It is not yet known how widespread CARNA 5 is among the numerous cucumoviruses that are less well characterized, including the more distantly related ones like peanut stunt virus which constitute a serious problem in the United States and elsewhere. We also do not know in which other CMV-related vegetable diseases CARNA 5 is the actual etiological agent. Nor do we know whether similar pathogenic RNA's are associated with other viruses. Many animal virus systems produce DI particles which contain smaller defective RNA's (*12*). As with CARNA 5 (*9*), the generation of such defective interfering particles is dependent on the type of host cells used for propagation (*15*). Plant viruses often produce and encapsidate subgenomic RNA components during replication (*5, 16*). RNA 4 of CMV is a good example of such a subgenomic RNA (*7*). In both defective interfering RNA's and subgenomic RNA's of plant viruses the nucleotide sequences are homologous with parts of the sequences of the viral genomes. However, CARNA 5 is different and very little if any of its nucleotide sequence is homologous with the genomic RNA's of CMV (*17*). This would place CARNA 5 in the category of satellite RNA's (*18*). However, as far as we know, a severe disease symptom like tomato necrosis, resulting from the presence of any of these types of RNA, has never been reported. Thus, the complete dependence of CARNA 5 on, and encapsidation with, the RNA's of the helper virus suggests that we are dealing with an insidious new form of "molecular parasitism" which expresses itself in two ways: in some hosts as an attenuation of disease symptoms while in others as a severe disease syndrome.

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