Research Note

Resistance to Tobacco Etch Virus in *Capsicum annuum*: Inhibition of Virus RNA Accumulation

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Received 21 April 1997. Accepted 10 June 1997.

Resistance to tobacco etch potyvirus (TEV) in *Capsicum annuum* cv. Dempsey is conferred by the recessive gene *et*^a. When Dempsey pepper plants were inoculated with either of two TEV isolates, virus did not accumulate in inoculated or noninoculated leaves. In contrast, each TEV isolate replicated to high levels in *C. annuum* cv. Jupiter, a susceptible parent of Dempsey pepper. To determine the basis of the resistance, Dempsey and Jupiter pepper plants were inoculated with a TEV isolate (TEV-HAT) engineered to express the β -glucuronidase gene (TEV-GUS). TEV-GUS moved cell-to-cell and systemically in infected Jupiter pepper plants. In contrast, there was no evidence of any single-cell infection foci induced by TEV-GUS in Dempsey pepper plants. Northern blot analysis of protoplasts inoculated with TEV-HAT RNA indicated that virus RNA did not accumulate in Dempsey pepper protoplasts, whereas TEV-HAT did replicate and accumulate in protoplasts from Jupiter pepper plants. Thus, the resistance to TEV conferred by the *et*^a gene is due to interference with virus RNA accumulation.

Potyviruses are some of the most prevalent and severe plant pathogens of *Capsicum* spp. worldwide. Consequently, much attention has been focused on identifying sources of resistance to potyviruses (McKinney 1952; Greenleaf 1956; Cook and Anderson 1959; Nagai and Smith 1968; Zitter 1972; Sowell and Demski 1977; Kuhn et al. 1989), but there is little information on the mechanisms responsible for the various resistance responses (Arroyo et al. 1996; Murphy and Kyle 1995).

Resistance to tobacco etch potyvirus (TEV) and potato Y potyvirus (PVY) in *C. annuum* PI 264281 was reported to result from either the pleiotropic effects of a single recessive gene or two closely linked recessive genes (Cook and Anderson 1959). Subsequently, a mutant of *C. annuum* cv. Yolo Wonder, designated YRP10, which was susceptible to TEV but resistant to PVY, was identified (Cook 1961). Inheritance studies on progeny from crosses of YRP10 and PI 264281 suggested

that the genes conferring resistance to PVY in both cultivars were allelic (*y*^a), implying that resistance to TEV in PI 264281 was conferred by a second closely linked recessive gene (*et*^a; Cook 1961). Recently, resistance to PVY in pepper homozygous for the *y*^a allele, introgressed from YRP10 into *C. annuum* cv. Yolo Y, was shown to be due to impaired cell-to-cell movement (Arroyo et al. 1996).

The TEV-resistance gene in PI 264281, *et*^a, was introgressed into *C. annuum* cv. Jupiter, resulting in *C. annuum* cv. Dempsey, which is homozygous for *et*^a (Lane et al. 1996). Dempsey pepper plants have resistance to TEV and *Xanthomonas campestris* pv. *vesicatoria* (races 1, 2, and 3), the causal agent of bacterial leaf spot (Lane et al. 1996). In contrast, Jupiter pepper plants are susceptible to both TEV and *X. campestris*. In field studies, TEV was not detected in Dempsey pepper plants, whereas 90% of the Jupiter pepper plants were infected with TEV (Lane et al. 1996). Similarly, after mechanical inoculation in the field, TEV was not detected in Dempsey pepper plants, but 100% of the Jupiter pepper plants were infected (Lane et al. 1996). To determine the basis of resistance in Dempsey pepper plants, replication of TEV was followed in both inoculated plants and protoplasts, using two isolates of TEV and a modified isolate of TEV carrying the β -glucuronidase (GUS) reporter gene.

Dempsey and Jupiter pepper plants were used in all experiments. TEV-HAT (a highly aphid-transmissible isolate; Allison et al. 1986) and TEV-GA (a Georgia isolate; Kuhn et al. 1989) were maintained on *Nicotiana tabacum* cv. Xanthi NN. TEV-GUS (TEV engineered to express the GUS reporter gene; Dolja et al. 1992) was maintained in *N. tabacum* cv. Xanthi (tobacco). TEV-HAT and TEV-GUS were obtained from J. Carrington (Washington State University, Pullman). Cucurbit mosaic cucumovirus (CMV; Alabama isolate) was maintained in *N. tabacum* cv. Kentucky 14. Homogenate from infected tobacco leaves was used as inoculum, except in experiments in which purified TEV-HAT or TEV-GA were used. Homogenate was prepared by grinding infected tissue with a mortar and pestle (1 g fresh weight of leaf tissue per 9 ml of inoculation buffer; 0.02 M KH₂PO₄, pH 7.0; 1 mM EDTA). TEV-HAT and TEV-GA were purified according to Dougherty and Hiebert (1980) from systemically infected tobacco leaf

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tissue and diluted in inoculation buffer prior to use. The two youngest leaves longer than 3.0 cm were inoculated on plants at the 4- to 6-leaf stage. Plants were maintained in the greenhouse under natural light conditions or in growth chambers (14 h of light/10 h of dark) at 24°C. TEV-HAT and TEV-GA infection was assessed by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's directions (Agdia Inc., Elkhart, IN).

Protoplasts were isolated from leaves of Dempsey and Jupiter pepper plants by overnight incubation in 0.45 M mannitol, 0.04% pectolyase, and 0.25% cellulase (Sigma Chemical Co., St. Louis) as described by Murphy and Kyle (1994). Protoplasts were inoculated by the polyethylene glycol (PEG) procedure (Loesch-Fries and Hall 1980) with minor modifications (0.5 ml of 40% PEG was added, followed by 5.0 ml of 0.45 M mannitol added dropwise). For each set of inoculations, 1×10^6 protoplasts were inoculated with viral RNA (10 µg of TEV-HAT or CMV RNA). The protoplasts were placed on ice for 15 min, the volume was brought up to 10 ml with 0.45 M mannitol (pH 5.47), and the protoplasts were

washed twice prior to suspension in 3.0 ml of 0.45 M mannitol inoculation medium (Murphy and Kyle 1994). Total RNA was extracted from protoplasts by an RNeasy plant mini-kit (Qiagen, Santa Clarita, CA), separated through a 1% (for TEV-HAT) or a 1.25% (for CMV) agarose gel containing formaldehyde (Sambrook et al. 1989), transferred to NYTRAN membrane (Schleicher & Schuell, Keene, NH), and cross-linked with a UV Stratagene 1800 (Stratagene, La Jolla, CA). Probes were ^{32}P -labeled cDNA prepared by reverse transcribing viral RNA with random hexanucleotide primers (Palukaitis 1988).

For immunoblot analysis, protein extracts from 1×10^5 protoplasts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 12.5% gels (Laemmli 1970) and electroblotted onto nitrocellulose. TEV-HAT or CMV coat proteins (CP) were detected with anti-TEV or -CMV polyclonal antisera and visualized by an alkaline phosphatase detection system (Murphy and Kyle 1994).

Dempsey pepper plants inoculated with TEV-HAT or TEV-GA prepared from infected tobacco did not develop symptoms up to 60 days postinoculation (DPI), and no virus was de-

Fig. 1. Histochemical localization of β -glucuronidase (GUS) activity in leaves of *Capsicum annuum* cvs. Jupiter and Dempsey plants inoculated with tobacco etch virus (TEV-GUS). **A**, Inoculated leaf from a Jupiter pepper plant at 4 days postinoculation (DPI); **B**, inoculated leaf from a Jupiter pepper plant at 8 DPI; **C**, noninoculated leaf from a Jupiter pepper plant at 12 DPI; **D**, inoculated leaf from a Dempsey pepper plant at 4 DPI; **E**, inoculated leaf from a Dempsey pepper plant at 8 DPI; and **F**, noninoculated leaf from a Dempsey pepper plant at 12 DPI.

tected in inoculated and noninoculated leaves by ELISA. In contrast, Jupiter pepper plants inoculated with TEV-HAT or TEV-GA showed systemic disease symptoms as early as 5 DPI, and virus was detected by ELISA in inoculated and noninoculated leaves (data not shown). Inoculated Dempsey pepper plants were tested further for TEV-HAT by inoculating extracts from the inoculated and noninoculated leaves onto Jupiter pepper and tobacco plants. No disease symptoms were detected on Jupiter pepper or tobacco nor was TEV-HAT detected by ELISA up to 30 DPI. In contrast, Jupiter pepper and tobacco plants inoculated with extracts from the inoculated and noninoculated leaves of Jupiter pepper plants previously inoculated with TEV-HAT showed disease symptoms by 5 to 6 DPI, and TEV-HAT was detected in inoculated and noninoculated leaves by ELISA (data not shown).

To determine whether resistance could be overcome with increasing virus concentrations, groups of 10 Dempsey and 10 Jupiter pepper plants were inoculated with 1, 10, 100, or 1,000 µg of purified TEV-HAT per ml. At each virus concentration, the inoculated Jupiter pepper plants showed symptoms by 5 DPI, and extracts from inoculated and noninoculated leaves tested positive for TEV-HAT by ELISA. No differences were detected in the time of appearance or symptom severity as a function of virus inoculum concentration (data not shown). In contrast, none of the Dempsey pepper plants developed disease symptoms at any of the inoculum concentrations used

by 30 DPI, and TEV-HAT was not detected in inoculated or noninoculated leaf tissue by ELISA, indicating the plants are highly resistant (data not shown).

The inability to detect TEV-HAT infection in Dempsey pepper plants could be due to a resistance mechanism that blocks cell-to-cell movement, virus replication, or earlier events, such as polyprotein processing. Virus movement was analyzed with TEV-GUS. Dempsey and Jupiter pepper plants were inoculated with TEV-GUS, and leaves from each cultivar were excised at 2, 4, 6, 8, 12, and 18 DPI for analysis by *in situ* histochemical GUS assays (Reimann-Philipp and Beachy 1993) to visualize sites of infection and subsequent cell-to-cell movement in inoculated and noninoculated leaves. Infection foci were visible by 2 DPI in inoculated leaves of Jupiter pepper (data not shown) and were continuing to expand at 4 DPI (Fig. 1A). At 8 DPI, extensive movement of TEV-GUS from initial infection foci had occurred throughout the vasculature of inoculated leaves (Fig. 1B), and by 12 DPI, GUS activity was detected in the noninoculated leaves of infected Jupiter pepper plants (Fig. 1C), indicating TEV-GUS had established a systemic infection. In contrast, no infection foci were detected in TEV-GUS-inoculated leaves of Dempsey pepper plants at 4 and 8 DPI (Fig. 1D and E) or as late as 12 and 18 DPI (data not shown) nor was virus detected in noninoculated leaves at 12 DPI (Fig. 1F). To determine whether TEV-GUS had established a subliminal infection in Dempsey pepper

Fig. 2. Inhibition of tobacco etch virus (TEV-HAT) accumulation in protoplasts from Dempsey pepper. Northern blot analysis of total RNA extracted from protoplasts inoculated with viral RNA purified from **A**, TEV-HAT or **B**, cucumber mosaic virus (CMV), respectively. Total RNA was extracted from 1×10^6 protoplasts at 30 h postinoculation, electrophoresed through a 1% agarose gel, and transferred to NYTRAN membrane. The blots were probed with 32 P-labeled cDNA of TEV or CMV RNA sequences to detect TEV and CMV RNAs. Immunoblot analysis of protein extracted from **C**, TEV-HAT- or **D**, CMV-inoculated protoplasts, respectively, at 24 and 48 h postinoculation. Protoplast extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis in a 12.5% gel and blotted onto nitrocellulose. The blot was probed with anti-TEV coat protein (CP) or anti-CMV CP polyclonal antisera (described in text). T indicates purified TEV RNA (**A**, 10 µg per lane) or purified TEV (**C**); J indicates extract from Jupiter pepper protoplasts; D indicates extract from Dempsey pepper protoplasts; C indicates purified CMV RNA (**B**, 10 µg per lane) or purified CMV (**D**); G indicates TEV-HAT genomic RNA; and 1, 2, 3, and 4 indicate CMV RNAs. The same results were obtained in a second independent experiment.

plants (i.e., virus replicated in initially inoculated cells but did not move cell-to-cell), TEV-GUS-inoculated leaves from Dempsey pepper plants that had been assayed for GUS at 2, 4, 8, 12, and 18 DPI were examined by light microscopy. No single-cell infection foci were detected, suggesting TEV-GUS does not propagate in Dempsey pepper.

To provide direct evidence that TEV-HAT did not accumulate in Dempsey pepper plants, mesophyll protoplasts from Dempsey and Jupiter pepper plants were isolated and inoculated with TEV-HAT RNA. Because Dempsey and Jupiter pepper plants are both susceptible to CMV (data not shown), protoplasts from plants of both cultivars were inoculated with CMV RNA as a positive control. Total RNA was isolated from protoplasts at 30 h postinoculation and analyzed by Northern blot hybridization. No viral genomic RNA was detected in protoplasts from Dempsey pepper plants inoculated with TEV-HAT (Fig. 2A), regardless of the length of time the Northern blot was exposed to film. In contrast, TEV-HAT did replicate in inoculated protoplasts from Jupiter pepper plants, as indicated by the presence of TEV-HAT genomic RNA (Fig. 2A), which comigrated with purified TEV genomic RNA (Fig. 2A). As expected, CMV replicated in both Dempsey and Jupiter pepper protoplasts to similar levels (Fig. 2B). The same results were obtained in a second independent experiment.

The results from the Northern blot data were supported by immunoblot data (Fig. 2C and D). TEV-HAT CP in inoculated protoplasts (1×10^5) from Jupiter pepper plants increased from 24 to 48 h postinoculation, whereas no TEV-HAT CP was detected in protoplasts from Dempsey pepper plants at either time point (Fig. 2C). Analysis of extracts from 7×10^5 inoculated protoplasts from Dempsey pepper plants also revealed no TEV CP (data not shown). Levels of CMV CP were similar in inoculated protoplasts from Jupiter and Dempsey pepper plants and were at near maximum levels by 24 h postinoculation (Fig. 2D).

The results indicate that the resistance mechanism in Dempsey pepper plants conferred by the *et^a* gene results from interference with virus production. This is different from the resistance mechanism reported for the *y^a* allele, which confers resistance to some PVY isolates that infect pepper (Arroyo et al. 1996). Resistance conferred by *y^a* is due to a block in cell-to-cell movement, which is indicated by the resistance functioning at the whole-plant level but not in inoculated protoplasts. The results presented here indicate that an additional mechanism in pepper, inhibition of viral RNA accumulation, provides resistance to potyviruses.

The mechanism of resistance in Dempsey pepper plants could be explained by a host product that interferes with the formation, assembly, or function of the viral polymerase. This type of mechanism may be similar to resistance controlled by the *Tm-1* gene in tomato or the *Ry* gene in *Solanum tuberosum* (potato). The resistance conferred by *Tm-1* to tobacco mosaic tobamovirus and *Ry* to PVY are expressed in protoplasts and whole plants and are characterized by suppression of viral RNA accumulation, albeit RNA accumulation can be detected at low levels (Yamafuji et al. 1991; Barker and Harrison 1984). Alternatively, the inability of TEV-HAT RNA to accumulate in Dempsey pepper could be due to inhibition of the protease activity required for processing the TEV polyprotein after translation of the genomic RNA. Resistance of this latter type is typified by a protease inhibitor described in *Vigna unguiculata* cv. Arlington (cowpea), in which processing of the cowpea mosaic comovirus 95-kDa polyprotein is blocked and viral replication is subsequently suppressed (Ponz et al. 1988). The *et^a* allele is similar to the *Tm-1*, *Ry*, and protease inhibitor resistance genes in that it is monogenic, but it differs in that it is recessive, whereas the *Tm-1* gene is semidominant and the latter two are dominant. Another approach to suppressing viral RNA accumulation is represented by the *Rx* locus in potato, which confers resistance to potato X potexvirus (PVX). *Rx*-mediated resistance is induced by structural features of the PVX CP (Köhm et al. 1993). However, the resistance is not specific to PVX, because potato plants coinfecting by PVX_{UK3} (a resistance-inducing strain) and CMV showed resistance to both PVX_{UK3} and CMV (Köhm et al. 1993). Dempsey pepper plants coinfecting with TEV-HAT and CMV remained resistant to TEV but were susceptible to CMV. In contrast, both viruses infected Jupiter pepper plants after coinoculation (data not shown). Therefore, the resistance mechanisms conferred by the *et^a* and *Rx* genes are likely fundamentally different.

The type of resistance described in Dempsey pepper plants has been designated "cultivar resistance" by Fraser (1990). In this type of resistance, the cultivar is resistant to a specific virus, whereas the plant species as a whole is susceptible to the virus. Therefore, not surprisingly, cultivar resistance may be overcome by virulent isolates of the virus. To this end, we have begun screening Dempsey pepper plants with different TEV isolates to identify a strain that overcomes the cultivar resistance. Characterization of a resistance-breaking strain will allow identification of the viral components associated with the resistance conferred by the *et^a* gene.

ACKNOWLEDGMENTS

This work was supported in part by state and Hatch funds allocated to the Georgia Agricultural Experiment Stations and by the Georgia Seed Development Commission and in part by the Auburn University Competitive Grants-in-Aid Program. We thank K. Lovins for technical support and J. Sherwood for critically reviewing this manuscript.

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