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Research Note

Impaired Cell-to-Cell Movement of Potato Virus Y in Pepper Plants Carrying the y^a ($pr2^1$) Resistance Gene

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The resistance mechanism to potato virus Y (PVY) in pepper plants homozygous for the recessive resistance allele y^a ($pr2^1$) was investigated. The results of the fluorescence immunochemical analysis of protoplasts prepared from virus-inoculated plants or virus-inoculated protoplasts indicate that this resistance is due to impaired cell-to-cell movement of the virus, rather than to an interference with virus replication.

Little is known about the nature of plant genes conferring virus resistance, although the genetics of virus resistance has been the subject of several comprehensive reviews (Fraser 1986; Fraser 1987; Fraser 1988; Fraser 1990). Both monogenic and polygenic resistances are known, but the former occur more often. Among these, most are the result of dominant alleles. The concept of resistance is in itself a complex one, covering the different manifestations of any type of restriction to a complete susceptibility to virus replication and disease development (Ponz and Bruening 1986).

Virus resistance genes can be functionally characterized through the analysis of their mechanisms of action. One of the best characterized examples is the resistance of Arlington cowpeas to cowpea mosaic comovirus (CPMV) (Beier et al. 1979; Kiefer et al. 1984; Ponz et al. 1988), in which an inhibitor of the proteolytic processing of the viral polyprotein has been found fulfilling the characteristics expected for a resistance mediator molecule (Ponz et al. 1988). In other cases, the analysis of resistance-breaking strains or mutants of cDNA infectious transcripts has provided insight into the resistance mechanisms. Through such analyses, different viral products have been implicated as mediators in different instances of resistance. Examples are the replicase (Meshi et al. 1988; Padgett and Beachy 1993), the cell-to-cell movement protein (Valkonen et al. 1991; Meshi et al. 1989; Calder and Palukaitis 1992), and the coat protein (Culver and Dawson 1989; Kavanagh et al. 1992; Köhm et al. 1993). In all these examples, the resistance is dominant or semidominant.

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Potyviruses constitute the largest group of plant viruses (Shukla and Ward 1989). Most examples of resistance to potyviruses are monogenic. Interestingly, a high percentage (almost 40%) of these are recessive traits (Provvidenti and Hampton 1992). From a mechanistic point of view the only example of potyvirus resistance characterized so far is the resistance to tobacco vein mottling virus (TVMV) found in the Tn86 cultivar of tobacco, in which the virus apparently cannot move between cells (Gibb et al. 1989).

Potato virus Y (PVY), the potyvirus type member, is an important pathogen of cultivated peppers, and of other members of the *Solanaceae*. We have studied the mechanism of the resistance against PVY in pepper, conferred by the recessive resistance allele y^a (Cook and Anderson 1959; Cook 1961), incorporated in several pepper cultivars, of which cv. Yolo Y is the reference one. Pepper-PVY isolates have been classified into pathotypes 0, 1, and 1-2 (Gebre Selassie et al. 1985). PVY isolates against which y^a confers resistance belong to pathotype 0, whereas pathotype 1-PVY isolates are able to break the resistance. These are not serologically distinguishable from those of pathotype 0 (Soto et al. 1994). So, the allele y^a (also called *vy1*, Gil Ortega et al. 1992, or $pr2^1$, Palloix and Kyle 1995) represents the first step in a series of resistance genes (possibly an allelic series, *vyn* or *pr2*) conferring resistance to different pathotypes of pepper-PVY. We show in this paper that the resistance conferred by y^a is based on impaired cell-to-cell movement of the virus.

Pepper (*Capsicum annuum* L.) cultivars Yolo Wonder (Y^aY^a) and Yolo Y (y^ay^a) used throughout this study were obtained from Ramiro Gil, Zaragoza, Spain. Pepper plants were mechanically inoculated with sap from tobacco (*Nicotiana tabacum* L. cv. Samsun NN) plants previously infected with pepper-infecting isolates of PVY (P-21-82 and P-62-81, Soto et al. 1994). Control plants were phosphate buffer-inoculated. PVY was purified according to Moghal and Francki (1976). PVY infection was assessed by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (Clark and Adams 1977), 21 days after inoculation using the monoclonal antibody (MAb) 10E3 from Ingenasa (Soto et al. 1994).

Protoplasts were prepared following Power and Chapman (1985) and Díaz et al. (1988), from pepper leaves collected 2 weeks after PVY inoculation with either PVY-0 or PVY-1.

For all the analyses after protoplast preparation their concentration was adjusted to 1×10^5 protoplasts per ml. For the immunocytochemical analysis, protoplasts were incubated with MAb 10E3 (1:1,000) in phosphate-buffered saline (PBS) for 2 h, and then incubated with sheep antimouse IgG conjugated with FITC (fluorescein isothiocyanate) diluted (1:40) in PBS. Fluorescent protoplasts were observed under a fluorescence microscope (Zeiss, Oberkochen, Germany) using the manufacturer-recommended set of filters for FITC (KP 490, FT 510, and LP 520). Protoplast viability was determined before immunolabeling, by treating aliquots with 0.01% fluorescein diacetate (FDA, Sigma, St. Louis, MO) and checking the proportion that fluoresced yellow-green (Larkin 1976). Protoplasts were inoculated with PVY-0 or PVY-1, essentially according to Potrykus et al. (1987), with slight modifications based on recommendations by Power et al. (1989).

Pepper epidermal strips were prepared from leaves collected 2 weeks after inoculation with either PVY-0 or PVY-1, and immunostained essentially as described (Luciano et al. 1989), except that the conjugate was with β-galactosidase and the substrate was X-gal.

PVY accumulation was determined by DAS-ELISA, as previously described (Soto et al. 1994). PVY-0 was not detected either in inoculated or in uninoculated leaves of Yolo Y after 21 days (Table 1). PVY-1 gave high readings both in Yolo Wonder and Yolo Y plants (data not shown). The evaluation of PVY accumulation at the cellular level was carried out in protoplasts prepared from leaves of susceptible and resistant pepper plants, 2 weeks after virus inoculation. These protoplasts were subjected to immunofluorescence analysis. The results are summarized in Table 2. Preparations from PVY-1 inoculated plants contained a high number of fluorescent protoplasts, indicative of a high level of virus multiplication, regardless of the pepper cultivar used. The same result was obtained in protoplasts from PVY-0–inoculated Yolo Wonder plants. Only a very small number of fluorescent protoplasts (approximately 0.06%) were found in preparations from the PVY-0–inoculated leaves of Yolo Y plants. The intensity of the fluorescence in these protoplasts was high and comparable to the intensity observed in Yolo Wonder–infected protoplasts. The immunofluorescence indicates similar levels of virus accumulation in infected cells of resistant and susceptible plants. No fluorescent protoplasts could be found in uninoculated leaves of Yolo Y plants. Figure 1 shows examples of photographs taken under UV light illustrating these results. A similar analysis was performed using pepper epidermal strips, instead of protoplasts. The results obtained after immunochemical staining (β-galactosidase conjugate and X-gal) of the strips were similar to the analysis of protoplasts (data not shown).

These results indicated that PVY-0 in Yolo Y plants was confined to some cells in the inoculated leaves and suggested that Yolo Y individual cells were able to support virus replication of both PVY-0 and PVY-1 pathotypes. This point was directly tested in experiments in which protoplasts from noninoculated Yolo Wonder and Yolo Y pepper plants were inoculated in vitro with PVY-0 or PVY-1, and the virus allowed to replicate for 36 h after inoculation. In all combinations, the number of virus-replicating protoplasts was high (80 to 85%), as revealed by immunofluorescence. An example of these fluorescent protoplasts is shown in Figure 2.

The phenotypic features of y^a-bearing PVY-resistant peppers, i.e., lack of symptoms and inability to detect virus multiplication by standard methods (ELISA), allow their classification as operationally immune (Beier et al. 1977; Ponz and Bruening 1986), since their reaction is indistinguishable from that of PVY-0 nonhost plant species. In spite of this apparent immunity, our results clearly show that PVY-0 is able to multiply in a small percentage of cells of a resistant $y^a y^a$ pepper leaf mechanically inoculated with the virus. In plants susceptible to PVY-0 used as positive controls most cells were virus-infected, and so were $y^a y^a$ plants inoculated with an isolate of pathotype 1. These results, together with the fact that individual infected cells coming from the resistant pepper cultivar show intensities of fluorometric stainings comparable to those of the susceptible cultivar (demonstrating similar levels of virus accumulation in them), indicate that the lack of success of PVY-0 isolates in infecting $y^a y^a$ resistant plants is due to the restriction of the viral short-distance cell-to-cell movement required for the systemic spread of the virus. This interpretation is further supported by the results obtained when individual protoplasts prepared from noninoculated PVY-0 resistant Yolo Y plants, were inoculated in vitro with PVY-0. Under these conditions, in which no virus movement between cells is required for infection, similar high percentages of immunofluorescent infected protoplasts were obtained regardless of the pathotype/cultivar combination used in the inoculation procedure. This supports the notion of unrestricted virus replication at the cellular level.

At least two additional mechanisms of resistance to PVY have been proposed in potato, a better-studied PVY host. The first one, mediated by the *Ry* gene, confers extreme resistance to the virus. This seems to act through an interference in the process of intracellular viral accumulation, since it is effective also in isolated protoplasts from resistant potatoes (Barker and Harrison 1984). The second mechanism has been found

Table 1. Enzyme-linked immunosorbent assay readings of samples from leaves of potato virus Y (PVY)–susceptible and PVY-resistant pepper plants, inoculated with buffer or a non-resistance-breaking virus pathotype

		PVY-0 inoculated	
Cultivars	Buffer inoculated[a]	Inoculated leaves[a]	Uninoculated leaves[a]
Yolo Wonder	0.085 to 0.120	1.750 to 2.000	2.000 to 2.500
Yolo Y	0.085 to 0.120	0.085 to 0.120	0.085 to 0.120

[a] The ranges shown contain values corresponding to four different inoculation experiments.

Table 2. Percentage of protoplasts showing virus replication in leaves of pepper cultivars Yolo Wonder and Yolo Y inoculated with two potato virus Y (PVY) pathotypes[a]

	Yolo Wonder		Yolo Y	
Leaves	PVY-0	PVY-1	PVY-0	PVY-1
Directly inoculated	70 ± 0.4[b]	60 ± 0.5	0.06 ± 0.03	65 ± 0.6
Systemically infected	80 ± 0.2	76 ± 0.6	0	70 ± 0.3

[a] Density 10^5 protoplats per ml. Viability 75%.

[b] Values represent the average percentage of infected protoplasts ± standard error. In each experiment 10^5 protoplasts were scored. The experiments were conducted four times.

in *Solanum brevidens*, a species in which PVY establishes a systemic infection of a very low viral titer (Gibson et al. 1990). Studies of viral replication in protoplasts from this relative of potato have shown complete cell susceptibility. However, the rate of progress of the infection is significantly lower in whole plants of this species when compared with PVY-susceptible potatoes (Valkonen et al. 1991). In contrast to this mechanism, $y^a y^a$ peppers restrict virus movement much more strongly.

A similar mechanism to the one described in this paper has been proposed for a different potyvirus, TVMV (Gibb et al. 1989), in a cultivar of tobacco (Tn 86) bred to incorporate the resistance to TVMV, TEV, and PVY originally found in the Virgin A Mutante tobacco mutant (Miller 1987). The resistance to PVY in this mutant has been shown to be inherited as a single recessive trait and the corresponding gene termed *va* (Koelle 1961; Gupton and Burk 1973). Apparently, this single recessive gene also conditions resistance to TVMV and TEV (Rufty et al. 1989). Further similarities or differences between the tobacco and pepper potyvirus resistance systems remain to be studied.

Short-distance cell-to-cell movement has been shown for several groups of plant viruses to involve the interaction of a viral movement protein with host components (Citovsky and Zambryski 1991; Citovsky and Zambryski 1993). For potyviruses, a detailed description of how this movement occurs is lacking. Recent results have shown that the P1 protein of some potyviruses exhibits nucleic acid–binding properties compatible with a role in mediating this type of virus movement (Brantley and Hunt 1993; Soumounou and Laliberté 1994). More direct evidence has been obtained for the involvement of the coat protein of TEV in viral short-distance cell-to-cell movement (Dolja et al. 1994; Dolja et al. 1995). Regardless of the specific viral products involved in the

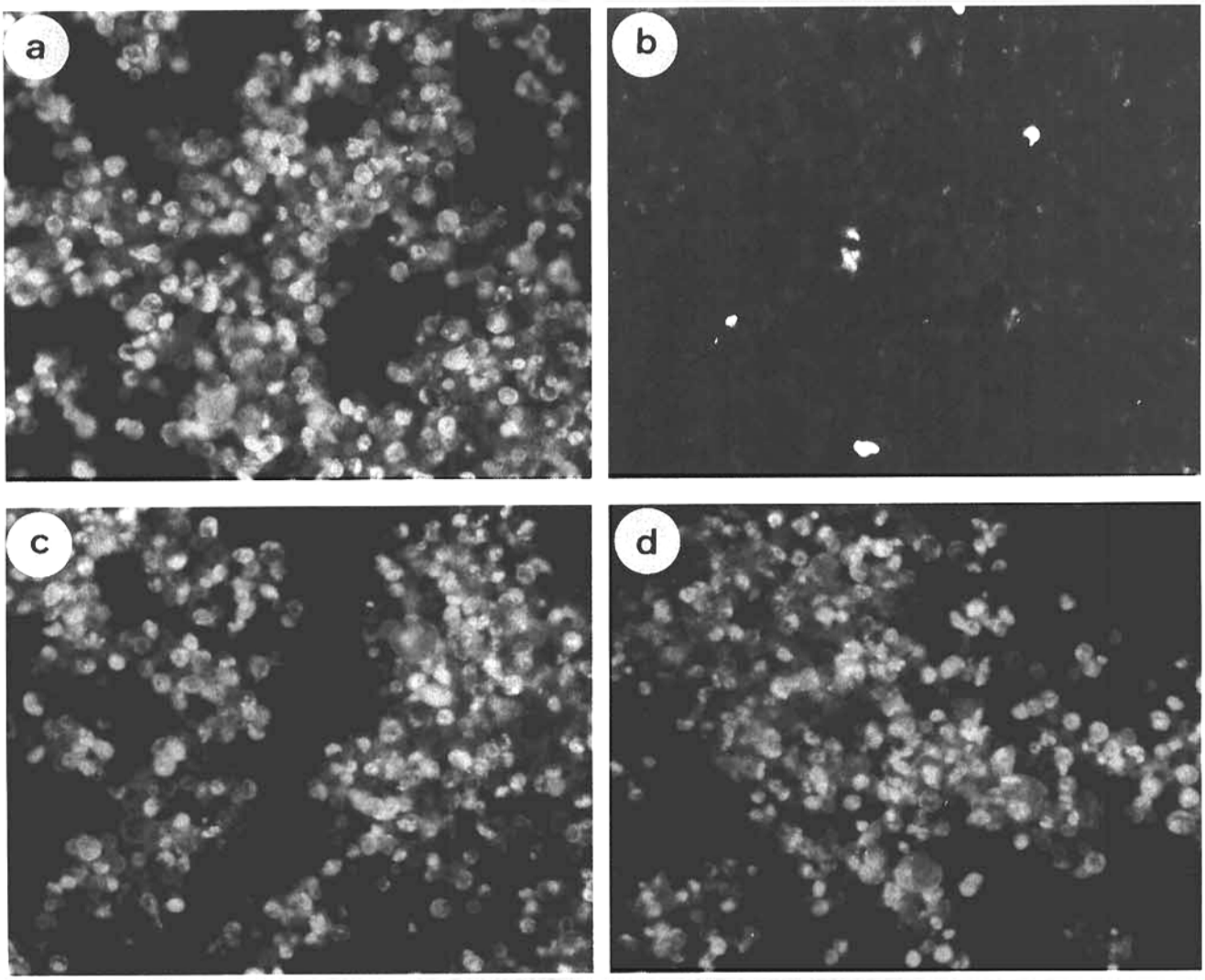

Fig. 1. Immunofluorescent detection of potato virus Y (PVY) in protoplasts prepared from PVY-inoculated pepper plants. Protoplasts were treated with a PVY-specific monoclonal antibody, which was revealed by means of a fluorescein isothiocyanate–conjugated sheep antimouse IgG. Photograph magnification is 200×. Protoplasts preparations from **A,** pepper cv. Yolo Wonder leaf directly inoculated with PVY-0; **B,** Yolo Y leaf directly inoculated with PVY-0; **C,** uninoculated (systemically infected) leaf of a PVY-0 inoculated Yolo Wonder plant; and **D,** uninoculated (systemically infected) leaf of a PVY-1 inoculated Yolo Y plant.

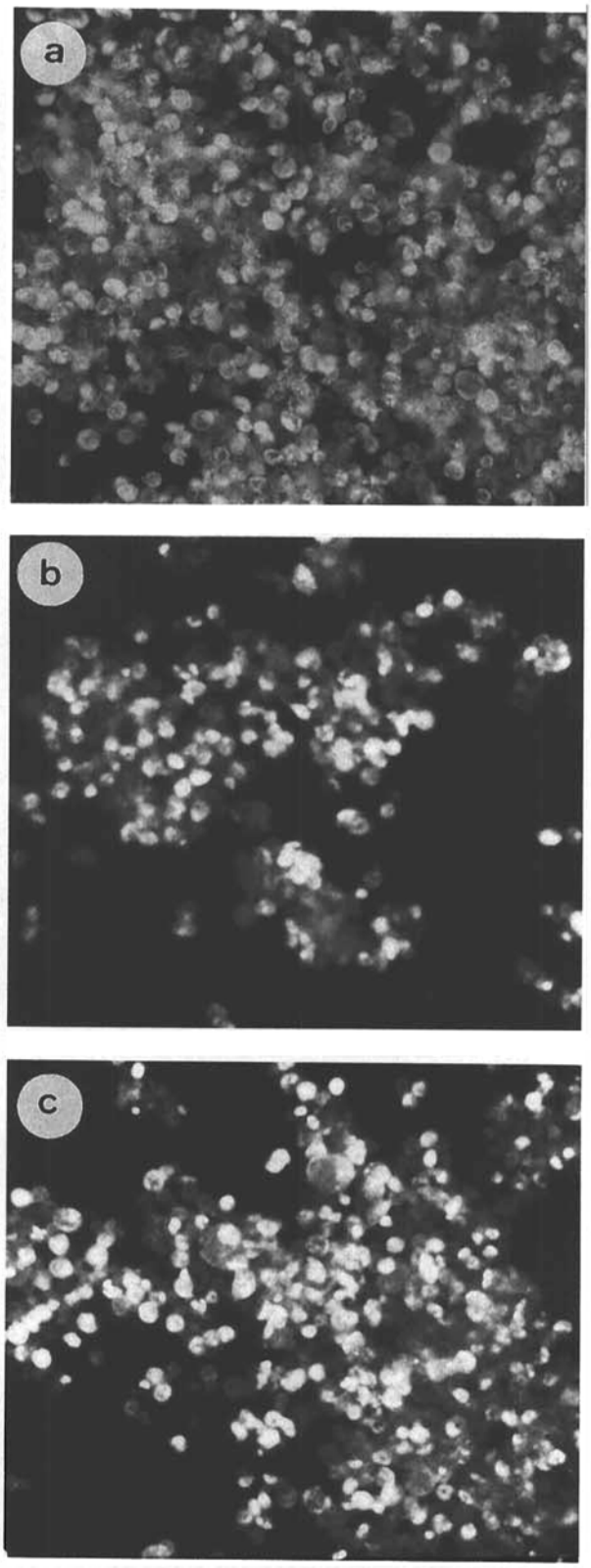

Fig. 2. Immunofluorescent detection of potato virus Y (PVY) in protoplasts inoculated in vitro. **A,** Pepper cv. Yolo Y protoplasts inoculated with PVY-0. **B,** Yolo Wonder protoplasts inoculated with PVY-0. **C,** Yolo Y protoplasts inoculated with PVY-1. Immunofluorescence analysis as in Figure 1.

movement, our results concerning the y^a allele strongly suggest an important role for some host component in the short-distance movement of PVY.

The y^a resistance and some instances of nonhost immunity to certain plant viruses described as subliminal infections in the early 1970s (Cheo 1970; Cheo and Gerard 1971), show mechanistic similarities in that the latter was shown to be due to the inability of the viruses to establish effective cell-to-cell movement (Sulzinski and Zaitlin 1982). The genetic basis of this nonhost immunity is not known; however, the similarity of the mechanisms involved is remarkable. The identification of host factors involved in the interaction with the movement protein of potyviruses is relevant to the study of proteins mediating resistance mechanisms like the one controlled by the y^a allele.

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