Research Note

Resistances to Turnip Mosaic Potyvirus in *Arabidopsis thaliana*

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The responses of a collection of *Arabidopsis thaliana* ecotypes to mechanical inoculation with turnip mosaic potyvirus were assessed. The virus induced characteristic severe symptoms of infection in systemically infected plants. Resistance was found in four ecotypes: Bay-0, Di-0, Er-0, and Or-0. Enzyme-linked immunosorbent assay results of the resistant ecotypes suggested that ecotypes Di-0, Er-0, and Or-0 actually consist of mixed genotypes with resistances acting at different levels in the virus life cycle. Another form of resistance was found in ecotype Bay-0, for which several lines of evidence indicated an interference with viral cell-to-cell movement in the inoculated leaves.

Plant model systems allow the development of reference points regarding fundamental hypotheses and models in many areas of plant biology. One of these areas deals with the study of the different types of interactions that can be established between a plant and a viral pathogen. The economic impact of the infection of crop plants by viruses has been a major driving force in the development of plant virology as a discipline (Agrios 1997; Brunt et al. 1996). At present, characterization of the role of virus gene products in the infection processes is much better than that of the plant factors involved in the interaction (Agrios 1997; Crute et al. 1997). For this reason, in the last few years the plant model system *Arabidopsis thaliana* has become a favorite for many research groups involved in the characterization of the plant side of the interaction with pathogens, including viruses (Kunkel 1996).

Potyriviruses form the largest family of plant virus pathogens and economic losses induced by them are significant worldwide (Shukla et al. 1994). Potyriviruses are single-stranded (+) RNA viruses whose virions are flexuous rods. The potyviral gene expression strategy occurs via the viral-controlled proteolytic processing of a single polyprotein precursor (Riechmann et al. 1992). The role of the different viral-encoded

proteins has been the topic of intense research efforts in the last decade (Kasschau et al. 1997; Schaad et al. 1997; and references cited therein).

We have previously identified turnip mosaic virus (TuMV) as a potyvirus able to systemically infect *Arabidopsis thaliana* (Martínez-Herrera et al. 1994). Tobacco etch virus (TEV), another potyvirus, also infects *A. thaliana* and sources of resistance have been identified to TEV in this species (Mahajan et al. 1998). TuMV has been ranked among the five most damaging vegetable viruses worldwide (Tomlinson 1987). The virus is extremely variable in its pathogenic and serological properties. Classification of a large collection of TuMV isolates into pathotypes according to their behavior in *Brassica* spp. has been proposed recently (Jenner and Walsh 1996). The translational initiation factor (iso) 4E of *A. thaliana* has been shown to interact specifically with the viral protein VPg (Wittmann et al. 1997). Several infectious cDNA clones of the type member of the pathotype 1 of TuMV, isolate UK-1, have been synthesized recently (Sánchez et al. 1998). As a necessary first step to gain an insight into plant functions involved in the potyvirus infection process, we describe in this paper the type of variability found in the interactions between the isolate UK-1 of TuMV and a large collection of *A. thaliana* ecotypes.

A collection of 106 ecotypes of *A. thaliana*, obtained from the Arabidopsis Seed Bank and from C. Somerville (Stanford University) was used for the initial screening. Prior to virus inoculation, seeds and plants were treated essentially as previously described (Martín Martín et al. 1997). Isolate UK-1 of TuMV, pathotype 1 (Tomlinson and Ward 1978; Jenner and Walsh 1996), was maintained by serial passages in plants of the susceptible *A. thaliana* ecotype Bs-1. The virus inoculum for the ecotype screening consisted of freshly prepared crude sap from TuMV UK-1 Bs-1 infected plants, obtained and used as described for oilseed rape mosaic virus (ORMV; Martín Martín et al. 1997). This type of inoculum was chosen instead of purified virus coming from different preparations for practical reasons of simplicity and reproducibility. Purified potyvirus virions are difficult to store long-term without a substantial loss of infectivity of the preparations (Brunt 1988). Some attempts to use such preparations showed this problem in the preliminary screenings.

Standard antigen-coated indirect enzyme-linked immunosorbent assay (ELISA; Koenig 1981) was used to analyze the inoculated plants 25 days after inoculation. A commercial

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anti-potyvirus monoclonal antibody kit (Agdia, Elkhart, IN) was used, following the conditions recommended by the manufacturer. The reaction of the substrate (*p*-nitrophenyl phosphate) was followed at 405 nm. Three different molecular approaches were used to evaluate the presence of accumulated viral RNA in plant tissue. (i) Plant skeleton hybridizations on whole *A. thaliana* plants were performed essentially as described (Leisner et al. 1992; Simon et al. 1992) with slight modifications. (ii) Northern (RNA) blot hybridization analyses (Sambrook et al. 1989) were performed on total RNA extracted from plant tissue. Equal amounts (1 µg) of denatured RNA samples were loaded and run in a formaldehyde-agarose gel, transferred to Hybond N⁺ membranes (Amersham, Little Chalfont, UK), hybridized at 65°C to a radiolabeled (Megaprime kit, Amersham) 515-bp cDNA fragment of the TuMV coat protein gene, obtained by immunocapture-reverse transcription-polymerase chain reaction (IC-RT-PCR, see below), and washed under highly stringent conditions. (iii) Detection of accumulated viral RNA in the total RNA preparations was performed by standard RT-PCR (Sambrook et al. 1989). IC-RT-PCR was carried out as previously described for potyviruses and other RNA plant viruses (Nolasco et al. 1993). Specific primers: 3'-(5'-ATGTATGGTCGGTCTTGGT TAC-3') and 5'-(5'-AGAGAAGGCAGAAAAGGAACGA-3'), corresponding to positions 69 to 584 of TuMV-coat protein sequence (Lehmann et al. 1997), were used to amplify a TuMV-specific, 515-bp cDNA fragment.

Protoplasts were obtained from leaves of *A. thaliana* plants that had been inoculated with TuMV. The protoplast preparations were done either 8 days after inoculation (protoplasts from inoculated leaves) or 21 days after inoculation (non-inoculated leaves). The overall protoplast preparation and immunochemical analysis were essentially as previously described for pepper protoplasts (Arroyo et al. 1996).

Symptoms that developed in infected plants of any susceptible ecotype were similar and included plant stunting and developmental arrest, leaf mosaic, sawed and curly leaves, and aborted flowers (Fig. 1). No necrotic lesions were seen on any

ecotype. The presence of visible symptoms in infected plants corresponded to a positive reading in ELISA (data not shown). Different ecotypes showed different percentages of infected plants, however. Individual plants that did not become infected after the first inoculation were reinoculated with the same type of inoculum, but no new infections were observed. Only 37 out of the 106 ecotypes showed a homogeneous response. The remaining 69 ecotypes presented different percentages of infected plants, ranging from nearly total infection (>90% infection rate) to nearly total resistance (around 10% infection rate). For this reason, all ecotypes analyzed were classified arbitrarily into three groups according to the percentage of individuals within ecotypes susceptible to TuMV infection (Table 1). The ecotypes in group III showed resistance to virus infection. In no plants of those ecotypes did we find symptoms of virus infection. ELISAs performed on non-inoculated leaves to determine whether systemic infection had occurred were negative (not shown).

The resistance found in the ecotypes in group III was further characterized. At least 200 plants of each ecotype were inoculated with TuMV UK-1. ELISAs were performed for all of them, differentiating inoculated and noninoculated leaves. The same number of plants of ecotype Bs-1 (group I) were processed in parallel as a positive control. Approximately two-thirds of the plants of ecotypes Er-0, Or-0, and Di-0 gave negative ELISA results both in inoculated and noninoculated leaves, indicating the absence of detectable levels of accumulated coat protein. The remaining one-third of the plants were ELISA positive, but only in the inoculated leaves, revealing a considerable amount of virus accumulation. This result suggests that different forms of resistance to the virus can be found in these resistant ecotypes, indicating the presence of variability for this trait within the ecotype. In contrast to the other three ecotypes, all plants of ecotype Bay-0 were negative by ELISA, either in inoculated or noninoculated leaves, 10 to 12 days after inoculation. Thus, ecotype Bay-0 behaved as a homogeneous genotype for resistance to infection by TuMV UK-1.

The resistance found in Bay-0 was characterized by several different approaches. The sensitivity of the ELISA used was

Fig. 1. Symptoms induced by turnip mosaic virus (TuMV)-UK1 in *Arabidopsis thaliana* plants. Sixteen-day-old plants of ecotype Do-0 were mechanically inoculated with TuMV-UK1. Arrowhead points to a mock-inoculated control plant. Picture was taken about 3 weeks after inoculation.

Table 1. Classification of *Arabidopsis thaliana* ecotypes according to TuMV-UK1 infections^a

Group	Infected plants (%)	Ecotypes
I	100	Bd-0, Be-1, Bor-0, Br-0, Bs-1, Can-0, Chi-0, Cit-0, Cl-0, Ct-1, Da-0, Db-1, Do-0, Eil-0, Fe-1, Ha-0, Is-0, Kil-0, La-er-, Lu-1, Lz-0, Mr-0, Mrk-0, Np-0, Ob-0, Old-1, Oy-0, Pf-0, Pr-0, Sue-0, Te-0, Uk-1, Wl-0
II	1 to 99	Aa-0, Ag-0, Ak-1, An-1, Ang-0, Bch-1, Bl-1, Bn-0, Bsch-0, Bus-0, Ca-0, Cal-0, Ci-0, Co-1, Col-0, Cvi-0, Dr-0, Edi-0, Ei-2, El-0, En-1, Ep-0, Et-0, Fi-0, Fr-2, Fr-3, Gd-1, Gr-1, Gü-0, Gy-0, Hau-0, Hi-0, Hs-0, Kä-0, Kb-0, Ko-2, Kr-0, Li-1, Lip-0, Lm-0, Ma-0, Mc-0, Me-0, Mt-0, Mz-0, Nc-1, Nd-0, No-0, Nw-0, Ove-0, Per-1, Pi-0, Pn-0, Po-0, Pt-0, Rd-0, RLD, Rsch-0, Sah-0, Se-0, Set-0, Sp-0, Ste-0, Stw-0, Tsu-0, Tul-0, Ty-0, Wc-1, Ws-0
III	0	Bay-0, Di-0, Er-0, Or-0

^a Indirect enzyme-linked immunosorbent assay (ELISA; anti-potyvirus antibody) was performed on noninoculated leaves of inoculated plants.

not sufficient to discard a low level of virus multiplication. In other situations, plant skeleton hybridization has been used in virus-inoculated *Arabidopsis* plants to visualize infection foci (Simon et al. 1992; Lartey et al. 1998). Thus, we performed this type of assay on the Bay-0 plants. As shown in Figure 2, we did not detect any hybridization signal with the viral-derived, radioactive probe in skeletons of Bay-0 plants in either inoculated or noninoculated leaves at 3 or 10 days after virus inoculation (Fig. 2C and D). This time period was sufficient to detect virus accumulation locally and systemically in skeletons of Bs-1 plants used as controls (Fig. 2B).

Total RNA from TuMV UK-1 inoculated leaves of Bay-0 was isolated 8 days after virus inoculation, and used in Northern blot hybridization analysis. Again, Bs-1 plants were used as controls. After only 3 h of film exposure of the blot hybridized to a radiolabeled viral cDNA probe, the autoradiograph gave a strong hybridization signal at the position corresponding to the mobility of TuMV RNA (9.6 kb) in the lane of susceptible ecotype Bs-1 (Fig. 3A), whereas no signal was

detected in the lane corresponding to the resistant ecotype Bay-0. However, when the same filter was overexposed (Fig. 3B), a faint band was observed in the lane of ecotype Bay-0 plants at the same position as for ecotype Bs-1. The mock-inoculated plants did not show any signal after 5 days of exposure (Fig. 3B). A quantitative comparison was done between signals of Bay-0 and Bs-1 plants, with electronic image analysis. After the appropriate corrections considering differences in exposure time (see Figure 3 caption), we estimated a 135-fold approximate difference in the level of accumulation between both ecotypes. This result strongly suggests that some very low level of virus replication and accumulation occurred in the inoculated leaves of Bay-0 plants. To confirm this suggestion, RT-PCR was performed on all three (virus-inoculated Bs-1 and Bay-0, and mock-inoculated Bay-0) RNA samples with TuMV-specific primers. An RT-PCR amplified cDNA fragment of the expected length (515 bp) was observed in the lanes corresponding to TuMV-inoculated Bs-1 and Bay-0 plants (Fig. 3C). No band was amplified in the control mock-inoculated Bay-0 sample. The possibility of a low level of virus accumulation (undetectable by Northern blot analysis) in the noninoculated leaves of TuMV-inoculated Bay-0 plants was tested by IC-RT-PCR. We did not detect any amplification band in extracts of Bay-0, whereas the corresponding 515-bp band was amplified in Bs-1 extracts (results not shown).

The above results obtained for ecotype Bay-0 are consistent with the conclusion that TuMV UK-1 is not able to invade the plant to produce a systemic infection under the conditions of the experimental system used. On the other hand, the faint signal obtained in the Northern blot hybridization analysis of the inoculated leaves and the band obtained after TuMV-specific, RT-PCR amplification in RNA samples from these leaves were indicative of a low level of virus replication and accumulation in the inoculated leaves. However, from these results we cannot exclude the possibility either that the viral RNA detected in these experiments corresponded to undegraded RNA from the virus inoculum used or that some inhibition of encapsidation or movement of this RNA was occurring, perhaps due to the lack of coat protein accumulation. Thus, we analyzed virus accumulation in protoplasts prepared from TuMV UK-1 inoculated *A. thaliana* plants of Bay-0, and Bs-1 as a control, using virus immunofluorescent detection with a TuMV UK-1 monoclonal antibody. Separated preparations of protoplasts from inoculated and noninoculated leaves were analyzed. The percentages of the different protoplast preparations showing fluorescence indicative of virus accumulation are shown in Table 2. Approximately 20% of the total amount of protoplasts prepared from inoculated leaves of the susceptible Bs-1 ecotype were intense green under the fluorescence microscope. The equivalent preparations from Bay-0 showed about 20 times less fluorescent protoplasts (about 1% of the total). The fluorescence exhibited by these protoplasts was visually as intense as in Bs-1 protoplasts, indicating that apparently there was not a detectable reduction in coat protein accumulation in these cells, but rather simply fewer infected cells. When protoplast preparations from noninoculated leaves of infected plants were examined, no Bay-0 fluorescent protoplasts were found, whereas more than half the protoplasts were infected in Bs-1 samples. Protoplasts from control mock-inoculated plants of ecotypes Bay-0 or Bs-1 gave no background fluorescent signal.

Fig. 2. Hybridizations of *Arabidopsis thaliana* plant skeletons with a turnip mosaic virus (TuMV)-specific probe. Skeletons of *A. thaliana* plants of ecotypes (A and B) Bs-1 and (C and D) Bay-0 were hybridized with a radioactive TuMV cDNA probe and film-exposed. Plant skeletons prepared (A and C) 3 and (B and D) 10 days after inoculation. Shapes of skeletons were pen-drawn on film to help in identification of signal localization. Arrows indicate inoculated leaves. B, Hybridization signals are only visible in some parts of skeleton corresponding to Bs-1 plants 10 days after inoculation.

The absence of difference in fluorescence intensity between infected protoplasts from the susceptible (Bs-1) and the resistant (Bay-0) lines is a strong indication that virus replication and accumulation take place in a small percentage of cells of the inoculated Bay-0 leaves, up to a level comparable to that found in susceptible ecotypes. Taken altogether, these results suggest that the cell-to-cell movement of TuMV UK-1 is strongly interfered with in Bay-0 plants. We (Arroyo et al. 1996) and others (Gibb et al. 1989; Valkonen and Somersalo 1996) have found this form of resistance to a potyvirus in different plant systems. As far as we are aware, none of the natural resistances to viruses described in *A. thaliana* to date act with this type of mechanism, although recently a chemically induced mutant of ecotype Col-0 has been obtained that resists infection by cucumber mosaic virus via a similar mechanism (Yoshii et al. 1998). Recent work has shown that the cell-to-cell movement of potyviruses is a complicated process involving several virus products (Dolja et al. 1994; Nicolas et al. 1997; Rodríguez-Cerezo et al. 1997; Rojas et al. 1997; Carington et al. 1998; Roberts et al. 1998). The identification of Bay-0 as an *A. thaliana* ecotype interfering with the cell-to-cell movement process may provide a useful tool for the eventual identification of plant factors involved in potyvirus movement.

The work in this paper deepens and extends our previous observation that TuMV is a pathogen of *Arabidopsis* (Martínez-Herrera et al. 1994). Isolate UK-1 of TuMV (Tomlinson and Ward 1978) was chosen because it is the type member of the pathotype 1 of this potyvirus and, as such, several sources of resistance in *Brassica* spp. are effective against it (Jenner and Walsh 1996). Given the considerable degree of relatedness between *Brassica* spp. and *A. thaliana* genomes (Cavell et al. 1998; Conner et al. 1998), we reasoned that the possibility of finding genes for resistance to TuMV in *Arabidopsis* would be high with this isolate.

Most *A. thaliana* ecotypes analyzed could be systemically infected with TuMV UK-1, showing severe symptoms without significant differences among the infected individual plants belonging to the different ecotypes. This was in high contrast to the results obtained by us in a parallel study conducted with

Table 2. Percentage of protoplasts showing virus replication in leaves of *Arabidopsis thaliana* ecotypes Bs-1 and Bay-0 inoculated with turnip mosaic virus (TuMV)-UK1^a

Leaf type	Bs-1		Bay-0	
	Buffer	TuMV	Buffer	TuMV
Inoculated leaves	0	20 ± 2 ^b	0	1 ± 0.4 ^b
Noninoculated leaves	0	55	0	0

^a Density 4×10^6 protoplasts per ml. Viability 80%.

^b Values represent average percentage of infected protoplasts ± standard error. Experiments were conducted four times. In each experiment approximately 10^5 protoplasts were scored.

Fig. 3. Analysis of viral RNA in resistant ecotype Bay-0. Ecotype Bay-0 and susceptible ecotype Bs-1 plants were inoculated with turnip mosaic virus (TuMV), and total RNA from inoculated leaves was prepared 8 days after inoculation. One microgram of each sample was analyzed by RNA blot hybridization with a ³²P-labeled, virus-specific probe (**A**, 3 h exposure; **B**, 5-day exposure) or (**C**) subjected to reverse transcription-polymerase chain reaction (RT-PCR) with TuMV-specific primers. **A** and **B**: Lanes 1–3, samples of mock-inoculated Bay-0, TuMV-inoculated Bay-0, and TuMV-inoculated Bs-1 (positive control) plants, respectively. Arrowheads point to position in gel of TuMV RNA (9.6 kb). Quantitative comparison between signal intensities from Bs-1 and Bay-0 RNA blot hybridizations was done by image analysis with Lane Manager 2.1 software (TDI, Madrid). Briefly, area of signal in lane 3A was delimited and its color intensity measured. Then, background from the corresponding position in lane 1A was subtracted. Value obtained was multiplied by 40 (difference in exposure time); resulting value was divided by number obtained after same procedure for the signal in lane 2B. **C**: Aliquots of RT-PCR products were analyzed in an agarose gel: lane 1, DNA molecular weight markers; lane 2, empty; lane 3, mock-inoculated Bay-0 plants; lane 4, TuMV-inoculated Bs-1 plants; lane 5, TuMV-inoculated Bay-0 plants. Arrowhead points to position in the gel of expected 515-bp product.

ORMV (Martín Martín et al. 1997). The different results obtained for these two viruses most likely relate to their different extents of dependence on the plant developmental stage to induce symptoms in the infected plants. Developmental resistance was described in *A. thaliana* for CaMV (Leisner et al. 1993). In addition, by comparison with the results obtained with ORMV, another important difference was that we did not find any TuMV UK-1 tolerant ecotypes.

A puzzling situation was obtained for ecotypes included in group II. Ecotypes in this group exhibited virus resistance upon mechanical inoculation. The percentages of resistant plants varied between ecotypes in the group. Again, this result is in contrast with those obtained for ORMV, where all the individual plants within a given ecotype showed the same type of symptoms and no resistant plants were found in susceptible ecotypes (Martín Martín et al. 1997). There are at least two possible explanations for this finding. (i) The type of inoculum used (crude sap from infected plants) was not sufficiently infectious to establish an infection in all the plants inoculated. However, the same inoculum was able to infect all the plants in many other ecotypes, in some of which, such as Bs-1 or RLD, literally hundreds or thousands of plants have been inoculated in our laboratory. This argues in favor of some ecotype-specific factor involved in the resistance. (ii) Resistance to mechanical inoculation has sometimes been reported for plant viruses (Jones 1993). If these individual plants within susceptible ecotypes are truly virus resistant, further characterization of the resistance would be worthwhile. We have only performed ELISA in noninoculated leaves of these plants, so we cannot discard the possibility that some type of interference with virus systemic movement is occurring. In any case, intra-ecotype variability seems to occur in group II ecotypes.

Four ecotypes showed resistance to TuMV UK-1 in all the inoculated plants (group III ecotypes). Approximately one-third of the plants in ecotypes Di-0, Er-0, and Or-0 showed an easily detectable level of virus accumulation in the inoculated leaves, but absence of virus in the noninoculated leaves, suggestive of a mechanism of interference with virus long-distance movement, a mechanism also found in *A. thaliana* for TEV, another potyvirus (Mahajan et al. 1998). This and other forms of resistance have been reported several times in *A. thaliana* for viruses of other taxonomic groups (Callaway et al. 1996; Dempsey et al. 1993; Lartey et al. 1998; Lee et al. 1994, 1996; Leisner and Howell 1992; Martín Martín et al. 1997; Simon et al. 1992; Takahashi et al. 1994; Wang and Simon 1997). As in group II ecotypes, intra-ecotype variability happens in these three ecotypes for the resistance trait.

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