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Response to potyvirus infection and genetic mapping of resistance loci to potyvirus infection in *Lactuca*

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Abstract We have investigated the interaction between two different potyviruses and resistant cultivars of *Lactuca sativa*. Turnip mosaic virus (TuMV) and lettuce mosaic virus (LMV) were used to inoculate several cultivars under different temperature regimes to characterize the resistance reaction. Resistance conferred by the recessive *mo* locus against LMV infection did not provide immunity. Virus accumulated in plant tissues to different levels depending on the genetic background of the cultivar, suggesting that several genes were involved in the resistance phenotype. Under temperature regimes that enhanced the hypersensitive reaction, resistant cultivars produced necrotic reactions. In contrast, resistance to TuMV infection conferred by the dominant *Tu* locus resulted in complete immunity in the plant. No virus accumulated in inoculated leaves nor was any necrotic reaction observed. The resistance loci were characterized at the genetic level by mapping them relative to molecular markers. Only weak linkages could be identified to *mo*, again supporting the hypothesis that several genes are involved. The *Tu* locus was mapped in two different crosses relative to several markers, the closest two linked at less than 1 cM. A high-resolution genetic map of the *Tu* locus was constructed by screening 500 F_2 individuals for recombinants around that locus.

Key words Disease resistance · Genetic mapping · Turnip mosaic virus · Lettuce mosaic virus

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Introduction

The incorporation of genetic resistance to viral pathogens into the germplasm represents the most efficient and cost-effective method of disease control in plants. Although in some cases genetic resistance can be overcome by mutations in the pathogen, some virus resistance genes have been used for decades without such mutants breaking the resistance mechanism (Duffus 1987; Taylor and Ghabrial 1986; Zaumeyer and Meiners 1975). It is likely that differences in the molecular mechanisms underlying different genetic resistances can explain the fact that some resistance genes can not be overcome by mutations in the pathogen. The genus *Lactuca* is infected by at least 24 plant viruses. Three viruses of the potyviridae, the largest and most economically important family of plant viruses, have been reported to infect that genus: lettuce mosaic virus (LMV), turnip mosaic virus (TuMV) and bidens mottle virus (BiMoV) (Provvidenti and Hampton 1992). All 3 viruses have overlapping host ranges but can nevertheless be differentiated on serological and host range grounds (Purciful et al. 1976). TuMV and BiMoV are able to infect almost all cultivars of lettuce (*L. sativa*).

In *L. sativa*, only four genes have been reported to confer resistance to different potyviruses: the *Tu* gene provides dominant resistance against TuMV infection (Zink and Duffus 1970), the recessive *mo* and *g* genes confer recessive resistance against LMV (Ryder 1970; Bannerot et al. 1969) and the *bi* gene confers resistance against BiMoV infection (Zitter and Guzman 1977). Both *Tu* and *mo* have been used to protect lettuce in commercial cultivars; the latter having been used in most California crisphead lettuce cultivars for over 20 years.

Few virus resistance genes have been cloned (Whitham et al. 1994), and our understanding of the series of cellular or molecular events taking place in the resistant plant cell is incomplete. We do not understand how the product of the virus resistance gene interacts

with viral components to prevent the virus from multiplying and/or moving in plant tissues. In most situations where resistance to potyviruses is conferred by a single gene in the plant, the resistance phenotype is dominant (Provvidenti and Hampton 1992). Fraser (1992, 1990) has proposed that products of dominant resistance genes actively interfere with viral processes; on the other hand, cells with recessive resistance genes would lack a cellular component necessary for virus multiplication and/or movement (a model described as passive resistance). Most models that attempt to explain resistance suggest that dominant resistance is an "active" process, where the plant synthesizes compounds that interfere with virus multiplication. Conversely, recessive resistance is the result of the plant cell lacking some factor(s) essential for the virus multiplication cycle. No such factor has yet been characterized, and the cellular mechanisms by which virus replication or spread is limited are unknown. Nevertheless, several hypotheses have been proposed based on knowledge of the interactions between different viruses and their hosts: resistance could take the form of inhibition of viral replication (Yamafuji et al. 1991), of polyprotein processing, since the potyvirus RNA is translated as one large polyprotein later processed into functional subunits (Bruening et al. 1987; Ponz et al. 1987), or of cell-to-cell movement of the virus (Nishiguchi and Motoyoshi 1987).

We report here the genetic and molecular interactions between lettuce mosaic (LMV) and turnip mosaic (TuMV) potyviruses and *Lactuca*. The resistance phenotypes to the 2 viruses have been characterized, which suggest distinct resistance mechanisms. The genetic localization of one of the genes (*Tu*) will allow its isolation and elucidation of the molecular mechanisms underlying that resistance to viral infection.

Materials and methods

Plant and viral materials

The TuMV isolate described by Tremblay et al. (1990) was propagated under greenhouse conditions on *Brassica perviridis*. The common-strain LMV isolate was obtained from the American Type Culture Collection (ATCC PV-63) and was propagated on 'Sabine' lettuce. Crude infected leaf extracts were prepared by grinding leaf tissue (0.25 g/ml) in 100 m*M* KH_2PO_4, pH 7.5, and used for inoculations. Plants were inoculated either by dusting the leaf surface with Carborundum (180 grit) and rubbing with a cotton swab saturated with crude extract from infected leaf tissue, or by spraying each leaf with an artist airbrush (Paasche) with a 4:1 mix of Carborundum (320 grit) and infected leaf extract. The crosses were provided by Drs. R. W. Michelmore (University of California at Davis), W. Waycott and E. J. Ryder (USDA, Salinas).

Immunoassays

An indirect enzyme-linked immunosorbent assay (ELISA) based on the protocol of Clark et al. (1986) was used for the detection of TuMV coat protein (CP) with a polyclonal TuMV CP serum (Robbins et al. 1994). All incubations were at room temperature or at 4°C. Wells were washed six times between each step using phosphate buffered saline (PBS; 137 m*M* NaCl, 2.7 m*M* KCl, 4.3 m*M* Na_2HPO_4, 1.4 m*M* KH_2PO_4, pH 7.0) containing 0.05% Tween-20 (PBST). Leaf samples to be analyzed were ground in extraction buffer (15 m*M* Na_2CO_3, 35 m*M* $NaHCO_3$ and 2% polyvinylpyrrolidone (PVP) 44 000) at a ratio of 0.05 g/ml of leaf in buffer. Samples were centrifuged for 1 min at 14 000 *g*. Microtiter plate wells (Falcon) were blocked with PBST containing 2% bovine serum albumin (BSA). Anti-TuMV CP serum, diluted 1:1000 in PBST buffer containing BSA (0.2%) and PVP (2%), was added to the wells, and antigen-antibody complexes were detected with goat anti-rabbit IgG alkaline phosphatase conjugate (Bio-Rad) diluted 1:5000 in the same buffer. Substrate solution containing para-nitrophenyl phosphate (1 mg/ml; Sigma) and diethanolamine (9.7%), pH 9.8, was added, and color intensity was measured at 405 nm.

Disease assays

To determine disease resistance phenotypes, we inoculated young lettuce plants with TuMV at the three-leaf to five-leaf stage with a crude extract of TuMV-infected *B. perviridis* (0.25 g leaf/ml 100 m*M* KH_2PO_4, pH 7.5). Using this procedure, 95% of the susceptible plants became infected in control experiments (data not shown). F_3 individuals from each family were inoculated and scored individually. Control plants were treated with sterile deionized water. ELISA was used to determine whether the inoculated plants were resistant or susceptible, in addition to scoring visual symptoms. The F_3 families were scored as all susceptible (progenitor F_2 plant was *tutu*), all resistant (progenitor was *TuTu*) or segregating for resistance (progenitor was *Tutu*).

Random amplified polymorphic DNA (RAPD) amplification

DNA was extracted from leaves using the cethylmethyl ammonium bromide (CTAB) method of Doyle and Doyle (1987). DNA amplifications were similar to those described by Robbins et al. (1994) and Williams et al. (1990). Polymerase chain reactions (PCR) were performed in 25-µl reaction volumes which contained 2–40 ng of genomic DNA, 0.3 µ*M* primer (decamer primers from Operon Technologies, California or University of British Columbia, Canada; Table 1), 100 µ*M* of each dNTP, reaction buffer (10 m*M* Tris-HCl, pH 8.8, 50 m*M* KCl, 15 m*M* $MgCl_2$, 1% Triton) and 1.5 units of *Taq* DNA polymerase (BRL). Amplifications were carried out in

Table 1 Sequence of the RAPD primers used in the genetic analysis of the *Tu* locus as well as the size of the polymorphic band identifying the marker. Only the previously unpublished markers are listed (see Robbins et al. 1994 and Witsenboer et al. 1995 for markers already published)

Primer	Sequence	Polymorphic band (bp)
UBC135	AAGCTGCGAG	240
UBC431A	CTGCGGGTCA	420
UBC431B	CTGCGGGTCA	940
UBC434A	TCGCTAGTCC	360
UBC434B	TCGCTAGTCC	1000
UBC439	GCCCCTTGAC	520
UBC448	GTTGTGCCTG	685, 750
OPL08	AGCAGGTGGA	410
OPL08	AGCAGGTGGA	1305

a thermal cycler programmed for 45 cycles of 1 min at 35°C, 2 min at 72°C, 1 min at 92°C, with an initial melt of 94°C for 5 min and a final cycle at 72°C for 4 min. Reaction products were separated on agarose gels.

Linkage analysis

Linkage analysis was performed using the MAPMAKER version 3.0 software (Lander et al. 1987). Linkage with a logarith of odds (LOD) score of at least 3 was considered significant. The best map orders and genetic distances were calculated from multipoint analysis. Chi-square analysis of segregation of *mo* was performed where the virus resistant progeny were scored as being homozygous for the recesssive (*aa*) genotype and susceptible progeny were scored as being either heterozygous or homozygous for the susceptible (*A_*) genotype.

Symptom development at different temperatures

Cultivars 'Sabine' (LMV susceptible), 'Floricos' (LMV resistant), 'Gallega' and 'Vanguard 75' were inoculated with LMV and later kept at different temperature regimes. All plants were grown at 22°C until inoculation. Infection was done between the third to fifth leaf stage. Plants were kept either at 22°C or transferred at 15°C or 32°C. After 5 days, the plants (3 per treatment) were shifted as to have nine different treatments. A similar design was used for infection of cvs 'Calmar' and 'Kordaat' with TuMV. Plants were exposed to temperatures of 15°C, 22°C, 32°C or 37°C for up to 35 days. Leaf tissue was kept frozen for ELISA analysis.

Results and discussion

Response to LMV infection

Resistance to LMV was incorporated into crisphead cultivars from wild *L. sativa* lines from Egypt (Ryder 1970). The gene conferring the resistance was named *mo* and was introduced into the 'Vanguard' cultivar to eventually form the near-isogenic line 'Vanguard 75' (Ryder 1970) by repeated backcrosses to the recurrent 'Vanguard' parent. A second recessive gene (*g*) was reported to control resistance to LMV infection in cv 'Gallega de invierno' (Bannerot et al. 1969). We have inoculated plants of cvs 'Sabine' (susceptible), 'Vanguard 75' (*momo*; resistant), 'Gallega' (*gg*, resistant) and 'Floricos' (*gg*, resistant). Virus was detected in all plants, with 'Vanguard 75' accumulating the least virus (0.06 ELISA units), 'Gallega' and 'Floricos' accumulating somewhat more virus (0.12 and 0.1 ELISA units, respectively) and 'Sabine' having the most virus (0.3 ELISA units). 'Sabine' is not known to carry any resistance genes to LMV and was expected to accumulate the most virus. Therefore, the resistance genes *g* and *mo* do not provide immunity but rather tolerance to LMV infection. Reduced infection levels, rather than immunity, in plants carrying the *mo* gene were also noted by others (Ryder 1976; Pink et al. 1992; Dinant and Lot 1992).

Initial reports of resistance to LMV in lettuce suggested that resistance was recessive on the basis of symptom severity (Ryder 1976). Monogenic resistance assessed using disease symptoms can be misinterpreted with resistance that is gene-dosage dependent. The latter is identified with resistance phenotypes that produce only partial localization of the virus (restriction to the inoculated leaf) or that decrease virus multiplication (Fraser 1990); in both cases, symptoms on inoculated leaves may not appear, but reduced amounts of virus would be detected immunologically.

Reports of symptoms of LMV infections on susceptible (*MoMo* or *Momo*) lettuce genotypes include leaf mosaic, blotching, vein clearing and leaf distortion (Tomlinson 1970). Local lesions, which have not been observed for LMV infections, are usually associated with dominant resistance genes. However, leaves from resistant 'Gallega' inoculated with LMV and maintained at 32°C afterwards for 5 weeks produced clearly visible local lesions (Fig. 1). Exposure of inoculated plants to high temperatures generally increases the size of the lesions produced in many plants. In some cases, a systemic infection is produced as virus spread is not restricted to the area around the lesion. More virus was found in plants maintained at high temperatures than in those maintained at 22°C or 15°C (data not shown), indicating that the partial resistance expressed in resistant lines can be overcome at high temperatures.

Linkage analysis to the *mo* locus

In order to characterize the *mo* gene at the genetic level, we have searched for DNA markers linked to the resistance phenotype. Three hundred decamer primers

Fig. 1 Necrotic lesions on *L. sativa* cv 'Gallega' induced by LMV on plants kept at 32°C. The hypersensitive response is more pronounced at high temperatures

were tested in RAPD assays for linkage to *mo*. The DNA used for the amplifications was from the near-isogenic lines 'Vanguard' (susceptible) and 'Vanguard 75' (resistant). Thirty primers were found to be polymorphic between the two lines and were then tested with cvs 'Dwarf 2' (*momo*) and 'Saffier' (*MoMo*). Only 16 of the 30 primers were polymorphic on that set of cultivars. This result is not unexpected since the near-isogenic lines may differ at more loci than the *mo* gene, and the amount of linkage drag around the *mo* locus has not been determined. The use of the second set of resistant/susceptible cultivars was to eliminate the markers linked to loci other than *mo*.

The 16 polymorphic markers were mapped using segregating populations from crosses between 'Dwarf 2' and 'Saffier' (57 F_2 individuals), and between 87-25M-1 (*momo*) and 87–1090M-1 (*MoMo*) (32 F_2 individuals from a population of 101). The F_2 individuals had been scored for their resistance phenotype after artificial inoculation under field conditions. Only the 32 individuals for which the phenotype had been verified twice were used from the 87-25M-1 X 87-1090M-1 cross. In cases where the polymorphisms were faint, Southern blots of the amplification products were probed with the labelled band corresponding to the polymorphic DNA marker.

Chi-square analysis taking each marker individually and testing segregation with *mo* was done in the two populations studied. In the 'Dwarf 2' and 'Saffier' cross, Chi-square analysis revealed that 12 out of the 16 markers selected were linked to *mo* at a confidence level of 95%. The closest marker to *mo* was UBC primer 551 at a distance of 28 cM. Only 3 markers (UBC688, UBC521a, UBC570) were segregating with *mo* in the 87–25M-1 and 87–1090M-1 cross at distances ranging between 40 and 48 cM.

The inability to detect tight linkage between DNA markers and a single recessive gene, as well as the formation of local lesions (a symptom usually associated with dominant resistance genes), can be interpreted as indicating that the resistance in cv 'Vanguard 75' is controlled by more than one gene. Michelmore et al. (personal communication) have recently identified a dominant gene for LMV resistance in some of the same cultivars used here. Ryder et al. (1993) noted that the level of resistance of a *momo* plant can be increased by incorporating (yet uncharacterized) other genes into the germplasm, indicating that perhaps several additional genes are involved and the resistance phenotype would therefore behave as a quantitative trait. Linkage analysis could be performed by pooling several individuals of each of the two extremene phenotypes (resistant and susceptible) and testing for polymorphisms between amplified DNAs.

Resistance to TuMV infection

Inoculation of TuMV on resistant cultivars at different temperature regimes did not produce local lesions, and the resistance remained effective at temperatures ranging from 15°C to 37°C. While virus accumulation in the susceptible cultivar ('Calmar') differed at different temperature regimes, no virus was detected in the 'Kordaat' cultivar. The *Tu* locus thus conferred immunity to TuMV infection.

Genetic mapping of the *Tu* locus

Zink and Duffus (1970) established that resistance to TuMV infection was controlled by a single dominant locus identified as *Tu*, and it was linked in repulsion with the *Dm5/8* locus (conferring resistance to the downy mildew pathogen *Bremia lactucae*) in a cross between cvs 'Calmar' (susceptible) and 'Kordaat' (resistant). Linkage between the 2 loci was estimated at 12.5 cM. Using a cross between the same parents, we mapped *Tu* relative to DNA markers identified by Michelmore et al. (1991) as linked to *Dm5/8* and to markers identified by Robbins et al. (1994) as linked to *Tu*.

Additional markers linked to the *Tu* locus were identified using RAPD markers (Table 1). The screen for polymorphic markers was targeted to the *Tu* locus by using a bulked segregant approach (BSA; Michelmore et al. 1991). Two pools of 8 homozygous individuals (*TuTu* or *tutu*) were created from F_2 individuals from a cross between cvs 'Calmar' and 'Kordaat' (Fig. 2). The genotypes of the individuals in the pools were obtained by determining the phenotype of 16 F_3 progenies from each of the F_2 parents. Allelic differences between the two pools of homozygotes should be linked to the *Tu* locus. From approximately 900 loci examined (from amplifications using 200 different decamer primers), 9 markers were found to be polymorphic between the two pools as well as between

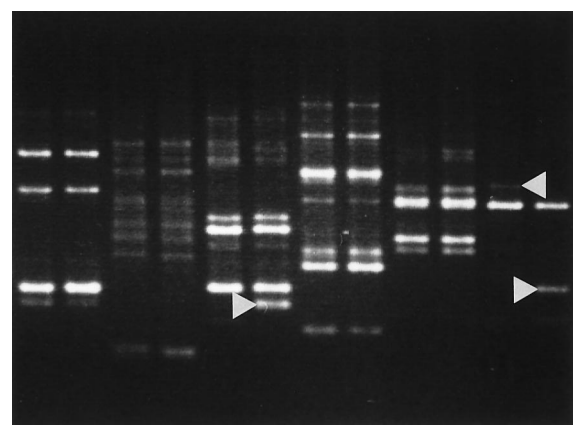

Fig. 2 Identification of RAPD markers linked to *Tu* using bulked segregant analysis. Each pair of lanes are PCR amplification products from one primer, using the susceptible (*left lane*) or the resistant (*right lane*) DNA pools as templates. The *arrowheads* indicate polymorphic bands

the two parents. The RAPD markers identified were all dominant. The markers were located using a cross between cvs 'Calmar' and 'Cobbham Green'. Three of those new markers (UBC431, UBC439, UBC135) are within approximately 5 cM of *Tu* (Fig. 3).

In order to improve the resolution of the genetic map and to verify the genetic location obtained above, we analyzed linkage between *Tu* and 10 of the previously mapped markers (UBC346, UBC517, UBC563, UBC599, OPD08, OPF12, OPL08, OPM18, OPX01, OPY13) in a second F_2 population derived from a cross between 'Calmar' and 'Kordaat' parents (Fig. 4). In addition, 2 new markers (UBC688 and UBC566) were added: those were identified using BSA and the resistant and susceptible pools formed from homozygous individuals from a cross between cvs 'Calmar' and 'Kordaat' (described above) (Fig. 4). Those 2 markers amplified a DNA fragment polymorphic between the two pools and the parents but could not be mapped in the 'Calmar' × 'Cobbham Green' due to the absence of polymorphism at that locus between the parents.

Although the 'Calmar' × 'Kordaat' map was generated using more F_2 individuals than the 'Calmar' × 'Cobbham Green' map (203 vs. 142 F_2 individuals), fewer recombinants were identified in the vicinity of the *Tu* locus. The *Tu* locus still mapped in the interval defined by OPD08 and OPU16, but too few recombinants were present to resolve *Tu* and some of the more tightly linked markers.

High-resolution mapping of the *Tu* locus

In the 'Calmar' × 'Cobbham Green' cross, OPL08 was perfectly linked to OPM18 as no recombinants could be identified that would separate the 2 markers. A cluster containing 5 markers (UBC517, UBC599, UBC439, OpY13, OpX01) also could not be resolved due to the absence of recombination. To increase the resolution of the map at the *Tu* locus, we sought additional recombinant individuals. Markers UBC431 and UBC135, which flank the *Tu* locus, were used to screen 500 F_2 individuals from another 'Calmar' × 'Kordaat' cross for recombination events. The 2 markers are linked in *cis*, and flank *Tu* with linkages of 4.1 cM and 1.4 cM, respectively (on the 'Calmar' × 'Cobbham Green' map). Thirty-three recombination events were detected between those 2 markers. The 33 additional individuals were used to map markers UBC431, UBC346 and UBC135 as well as *Tu*. The phenotype for these 4 marker loci were determined by RAPD amplification using F_2 genomic DNA from the recombinant individuals; the genotype at the *Tu* locus was determined by infecting 6–14 F_3 individuals from each of the 33 F_2

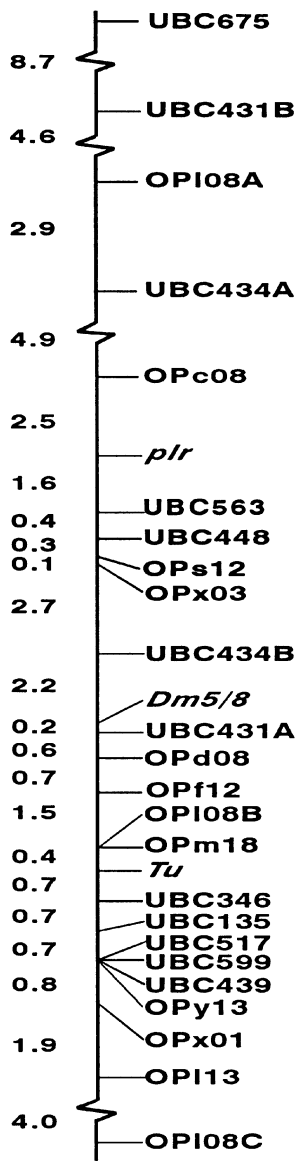

Fig. 3 Genetic linkage map of *L. sativa* in the region of the *Tu* locus. The mapping population consisted of 142 F_2 individuals from a cross between cvs 'Calmar' and 'Cobbham Green'. Marker loci are listed on the *right* and genetic distances in centiMorgans on the *left*. *Tu*, *Dm5/8* and *plr* are resistance genes against TuMV, downy mildew (*Bremia lactucae*) and *Plasmopara lactucae-radicis*, respectively

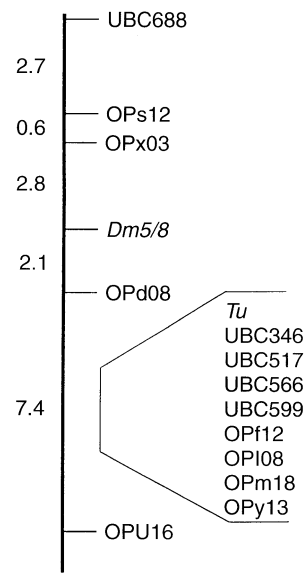

Fig. 4 Genetic map of the *Tu* locus conferring resistance to TuMV infection in *Lactuca*. The mapping population consisted of 203 F_2 individuals from a cross between cvs 'Calmar' and 'Kordaat'. Twelve individuals were recombinant in this region, which includes 13 RAPD markers linked to two resistance genes (*Dm5/8* and *Tu*). The genetic distances between loci are shown on the *left* and are expressed in centiMorgans

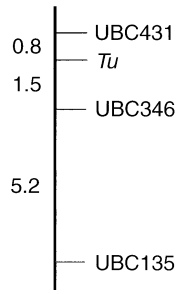

Fig. 5 High-resolution linkage map of the *Tu* locus. The mapping population consisted of 500 F_2 individuals derived from a 'Calmar' × 'Kordaat' cross. Marker phenotypes were obtained from F_2 individuals, which were also genotyped using F_3 progeny for their resistance or susceptibility to TuMV infection

parents and assessing resistance or susceptibility both visually and by ELISA immunodetection of the virus CP. The resulting high-resolution map of the *Tu* locus (Fig. 5) shows that the UBC431-UBC135 interval has now expanded to 7.8 cM, from 4.6 cM in the 'Calmar' × 'Cobbham Green' map, using 142 F_2 individuals.

The maps developed here, along with the individuals with recombination events flanking the *Tu* locus, will allow physical mapping of the locus using probes for that genomic region. The markers identified in this work will facilitate the identification of DNA clones physically linked to the resistance locus. A cloned *Tu* gene will allow the study of the resistance mechanism at the molecular level.

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