Several Genes in \textit{Lycopersicon esculentum} Control Hypersensitivity to \textit{Xanthomonas campestris pv. vesicatoria}

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\textbf{ABSTRACT}


Resistance to tomato (\textit{Lycopersicon esculentum}) accession Hawaii 7998 (H7998) to bacterial spot caused by \textit{Xanthomonas campestris pv. vesicatoria} is associated with a hypersensitive reaction. In F$_2$ plants from a cross of H7998 with the susceptible tomato genotype Florida 7060, the time between infiltration of leaves with a high-inoculum concentration ($5 \times 10^7$ cfu/ml) and the appearance of necrosis and bacterial growth after infiltration of leaves with a low-inoculum concentration ($5 \times 10^5$ cfu/ml) were intermediate to the two parents. The times for confluent necrosis among plants in the F$_2$ population injected with a race 1 strain or with a race 2 strain converted to avirulence with the \textit{avrRsv} gene ranged from those of the susceptible parents to those of resistant parents but included intermediate times and clearly showed continuous distribution. We hypothesize that hypersensitivity like that of H7998 is controlled by three recessive genetic loci. Seven genotypes from a segregating backcross population that had different times to confluent necrosis after inoculation were asexually propagated and inoculated. Electrolyte leakage and bacterial growth in leaf tissue of the genotypes were correlated with ratings for necrosis, and different groups of plants could be distinguished. The hypersensitive reaction in H7998 is controlled by more than one gene and does not appear to fit the gene-for-gene hypothesis.

Bacterial spot of tomato, caused by \textit{Xanthomonas campestris pv. vesicatoria}, is a serious disease in regions where warm temperatures and heavy rainfall occur together during the growing season (10). Protection of plants from the disease by chemical sprays is inefficient, and resistant tomato cultivars have not been developed. Scott and Jones (11) reported one tomato genotype, Hawaii 7998 (H7998), that has a high level of field resistance to bacterial spot. There is hope for effective control of this disease in the future with the successful transfer of resistance from H7998 to commercial tomato cultivars (13). Resistance to bacterial spot in H7998 is associated with a hypersensitive reaction to the pathogen (6). In other plants, resistance, which is associated with a hypersensitive reaction, follows the gene-for-gene hypothesis (7). According to this hypothesis, the incompatible interaction between products of an
avirulence gene in the pathogen and a complementary resistance gene in the plant results in a hypersensitive reaction (4). In most cases, the avirulence and resistance genes have been shown to be dominantly inherited (2,7).

Wang et al. (15) identified an X. c. vesicatoria strain that does not cause a hypersensitive response in H7998 and designated it race 2. In other work, it was determined that the avirulence gene, avrRxv, cloned from DNA of a race 1 strain of X. c. vesicatoria converted a race 2 strain to race 1 (16). A 680-bp region of avrRxv, amplified by polymerase chain reaction, was present in all strains of race 1 but was not present in strains of race 2 (1). Resistance to bacterial spot in H7998 is inherited quantitatively under field conditions (12). Because the quantitative inheritance observed in the field may result from variations in environmental factors or from other mechanisms not related to hypersensitivity in H7998, studies of the inheritance of the hypersensitive reaction in the progeny of H7998 were initiated to determine the role of the hypersensitive reaction in bacterial spot resistance.

Here we report on a novel hypersensitive reaction in H7998 to race 1 strains of X. c. vesicatoria determined by using progeny crosses from crosses between H7998 and lines of Lycopersicon spp. susceptible to bacterial spot. We also present data on the introduction of an avirulence gene, avrRxv, into an X. c. vesicatoria race 2 strain, which changes the strain from virulent to avirulent on H7998, and the interaction of this gene with more than one resistance gene in H7998 for expression of the hypersensitive reaction.

**MATERIALS AND METHODS**

Plant materials and growth of plants. Resistant parent H7998 was crossed with susceptible parents Florida 7060 (Fla. 7060) and L. pennelli accession LA716, after which F1 seed was obtained from each F1.

Seed of parents, F1, and F2 were planted in a plug-mix (W. R. Grace & Co., Cambridge, MA), and the resulting seedlings were transferred to Metromix 300 (W. R. Grace & Co.) in 10-cm plastic pots. Growth of seedlings occurred in a greenhouse at temperatures of 25°C (night) to 35°C (day). A soluble 20-20-20 fertilizer (Peters Fertilizer Products, W. R. Grace & Co., Fogelsville, PA) was added to the pots in the amount of 0.4 g per pot every 2 wk. For most tests, the main stem of each seedling was removed above the fully expanded fourth true leaf in tests for ratings of necrosis; however, for analyses of internal bacterial populations and electrolyte leakage, plants were transplanted after growth for 4 wk to 15-cm plastic pots, and about 1 wk later the main stem was removed above the fully expanded sixth true leaf. Approximately 7 days after they were topped, plants were inoculated and then transferred to a growth chamber kept at 24°C with a daily 16-h light period.

**Bacterial strains and inoculum preparation.** Strains 75-3 and 90-14 of race 1 of the tomato group of X. c. vesicatoria, which induce a hypersensitive response in H7998 and a susceptible reaction in Fla. 7060, were used in this study, XV56(pXV9006), a strain of X. c. vesicatoria race 2 that contains pXV9006 (a plasmid containing avrRxv[14,16]), was received from M. Whalen (University of California, Berkeley). The strains were stored in sterile tap water.

Strains were subcultured for 48 h on nutrient agar, and a single colony of each strain was transferred to nutrient broth for growth, except XV56(pXV9006), which was subcultured and grown in nutrient broth in the presence of 10 μL of tetracycline per milliliter. Cultures were shaken overnight at 30°C and centrifuged. The pellets were resuspended in sterile tap water, and the suspensions of bacteria were standardized to 0.3 (a concentration equal to 5 × 10^8 cfu/ml). Suspensions of lower concentrations were obtained by dilution of the standard suspension.

**Plant inoculation and disease ratings.** Inoculum (5 × 10^8 cfu/ml) was infiltrated into about 1 cm² of leaf area in tests for ratings of necrosis. Infiltrations were accomplished by forcing suspensions of cells into the intercellular spaces with a hypodermic syringe and a 26-gauge needle. The percentage of the total infiltrated area that was necrotic was estimated every 8 h after infiltration. The following rating scale was used: 0 = no necrosis; 1 = 1–20%; 2 = 21–40%; 3 = 41–60%; 4 = 61–80%; and 5 = 81–100% (confluent necrosis) in the infiltrated tissue.

An area of approximately 2 cm² of a leaflet of the most recently developed mature leaf was infiltrated with a bacterial suspension containing 5 × 10^8 cfu/ml in tests to determine populations in inoculated leaves. Bacterial populations were determined on each of three leaflets daily for 5 days by a dilution plate method according to the procedure described previously (5).

Inoculum consisting of 5 × 10^8 cfu/ml was infiltrated into approximately 5 cm² of leaf area for assays of electrolyte leakage. Electrical conductivity of water in baths containing inoculated tissues was determined by the procedure previously described (5) with samples taken every 12 h after infiltration. Each treatment was replicated three times from each of three leaflets from the same plant.

**Inheritance studies.** Ratings of necrosis over time after inoculation with strains 90-14 and XV56(pXV9006) were compared for reactions in nine plants of each parent (H7998 and Fla. 7060), 15 F1 plants, and 129 F2 plants. Bacterial suspensions were infiltrated into leaflets on the opposite sides of the same leaf. Inheritance data were analyzed for the first time period when H7998 was 100% necrotic and Fla. 7060 had no necrosis.

**Stability of disease reactions.** Seven plants of a backcross population, H7998 × (H7998 × L. pennelli LA 716), that had different times to confluent necrosis after inoculation were selected. These plants were placed in 15-cm pots, and after growth, cuttings of each plant were rooted in plug-mix and cultured as above. The axenically propagated plants were inoculated with strain 75-3 for determinations of electrolyte leakage and for assays of bacterial growth as described above. Each assay was replicated three times on each of three leaflets on the same leaf. The relationships of necrosis ratings (electrolyte leakage values) and populations of bacteria were analyzed by regression with SAS computer software (SAS Institute, Cary, NC).

**RESULTS**

**Disease reactions in F1 plants.** The F1s, H7998, and Fla. 7060 plants differed in disease reaction after leaflets were infiltrated with strain 90-14. The elapsed time between the start of necrosis and confluent necrosis was longer for F1 plants than for either parent (Fig. 1A). In addition, the time required for the beginning of necrosis and the time for confluent necrosis differed for each

![Fig. 1. A. Necrosis development and B. population dynamics of Xanthomonas campestris pv. vesicatoria over time in parents and F1 progeny of a genetic cross between Hawaii 7998 and Florida 7060 tomato. The following necrosis rating scale was used: 0 = no necrosis; 1 = 1–20%; 2 = 21–40%; 3 = 41–60%; 4 = 61–80%; and 5 = 81–100% (confluent necrosis). Vertical lines represent one standard deviation.](Vol. 84, No. 7, 1994 703)
TABLE 1. Frequency distribution of tomato plants in an F_{3} population of Hawaii 7998 (H7998) × Florida 7060 (Fla. 7060) according to ratings for necrosis over time of leaves infiltrated with a bacterial suspension (5 × 10^{5} cfu/ml) of strain 90-14 of Xanthomonas campestris pv. vesicatoria

<table>
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<tr>
<th>Hours</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>H7998</th>
<th>F_{1}</th>
<th>Fla. 7060</th>
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<tr>
<td>16</td>
<td>124</td>
<td>4</td>
<td>1</td>
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<td>24</td>
<td>112</td>
<td>15</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>3-5</td>
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<td>12</td>
<td>112</td>
<td>5.0</td>
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</tbody>
</table>

*Hours after infiltration.
*0 = No necrosis; 1 = 1-20%; 2 = 21-40%; 3 = 41-60%; 4 = 61-80%; and 5 = 81-100% (confluent necrosis). Numbers in each column represent F_{3} plants out of 129 that had the indicated rating.
*Range of necrosis ratings for nine plants.
*Range of necrosis ratings for 15 plants.

Plant type. A curve representing the increase of bacteria in leaves of F_{3} plants was intermediate to the curves for the two parents (Fig. 1B).

Segregation of hypersensitivity in leaves in an F_{3} population.
The time for confluent necrosis varied from 32 to 40 h in H7998 and from 96 to more than 112 h in Fla. 7060 (Table 1). The time for confluent necrosis in the F_{3} population varied between the times required for confluent necrosis of the two parents. Three of 129 plants had confluent necrosis by 40 h after inoculation. Ratings of necrosis varied among the plants over time, and no discrete segregation indicative of monogenic control was evident at the time period when H7998 was 100% necrotic and Fla. 7060 had no necrosis.

Confluent necrosis was observed in plants of H7998 32-48 h after inoculation with the transconjugant XV56 (pXY9006) (data not shown). The average ratings of necrosis in all plants of H7998 to both strains at each rating period were similar. However, in all plants of Fla. 7060, the average ratings for necrosis were higher at each rating period after 56 h in leaflets infiltrated with the transconjugant than in leaflets infiltrated with the wild strain.

From the data of F_{1}, F_{2}, and the parent plants 40 h after being infiltrated with strain 90-14 (Fig. 2A) or with strain XV56 containing avrRxv (Fig. 2B), it is clear that hypersensitivity, as measured by necrosis at the time when H7998 was 100% necrotic and Fla. 7060 had no necrosis, is largely controlled by recessive gene action. Most F_{2} plants were similar to Fla. 7060, although a few plants showed a low level of hypersensitivity. This is reflected in the F_{2} data, where a high percentage of the plants were rated 0 (49%) and a low percentage were rated 5 (<3%). The F_{2} data had a continuous distribution with intermediate hypersensitivity levels not present in the parental or F_{1} generation.

It appears that the inheritance of hypersensitivity is controlled by more than one gene. A quantitative genetic analysis was not done because of the absence of backcross generations. Nevertheless, we hypothesize that hypersensitivity is controlled by three loci. Under the model, plants with dominant alleles at either two or three loci would appear like Fla. 7060 or the F_{1}; plants with dominant alleles at one locus would have intermediate hypersensitivity; and plants with all recessive alleles would have rapid hypersensitivity like H7998. These classes would segregate 54:49:1, respectively. Chi-square tests of this hypothesis indicated an acceptable fit for both 90-14 or XV56 containing avrRxv (Table 2).

Confirmation of the model would require progeny testing in which F_{2} populations that rated 5 would be expected to breed true and the segregates with intermediate hypersensitivity would be expected to segregate three intermediate to one hypersensitive. It should be noted that other variations of a three-gene model also had acceptable fits in chi-square tests. A 27:36:1 ratio of 0:1:4:5 necrosis classes fit at P = 0.5-0.9, as did a 27:27:9:1 ratio of 0:1:2:3:4:5 or 0:1:2:4:5 necrosis classes for strains 90-14 and XV56, respectively. Each of the three-gene models requires certain assumptions that are not entirely satisfactory, and none can be proven unequivocally. However, with the present data, a three-gene model is the best explanation available. We also pooled all plants with hypersensitivity ratings greater than those of the F_{1} population and tested 3:1 ratios, which provided an acceptable fit only for the data from strain 90-14 (χ² = 2.81, P = 0.05-0.1). The fits in the experiments were not acceptable (P < 0.005) for a 3:1 ratio were XV56 (pXY9006) or the combined data were used. Epistatic ratios such as 12:3:1 or 9:7 were not acceptable either.

Stability of disease reactions. The time to confluent necrosis after inoculation varied among plants of an F_{3} population from the interspecific cross of H7998 and L. pennellii accession LA 716 (data not shown). The results were similar to those for the F_{1} population of the interspecific cross (Table 1). Variation in necrosis also occurred among progeny of the backcross of an interspecific F_{1} plant and a plant of H7998 (data not shown).

Among seven genotypes selected for differences in disease reaction from the interspecific backcross progeny, differences in electrolyte leakage occurred at 24, 36, and 48 h after inoculation. There was a significant linear relationship between bacterial
TABLE 2. Segregation of Hawaii 7998 (H7998) × Florida 7060 (Fla. 7060) F₂ generation for hypersensitivity to two strains of Xanthomonas campestris pv. vesicatoria, 90-14 and XV56(pXV9006), and chi-square tests for genetic control by three genes

<table>
<thead>
<tr>
<th>Strain</th>
<th>Total plants</th>
<th>Susceptible</th>
<th>Intermediate</th>
<th>Hypersensitive</th>
<th>Expected ratio</th>
<th>$x^2$</th>
<th>$P$</th>
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<tr>
<td>90-14</td>
<td>129</td>
<td>105</td>
<td>21</td>
<td>3</td>
<td>54.9:1</td>
<td>1.06</td>
<td>0.5-0.9</td>
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<tr>
<td>XV56(pXV9006)</td>
<td>129</td>
<td>113</td>
<td>12</td>
<td>4</td>
<td>54.9:1</td>
<td>4.18</td>
<td>0.1-0.5</td>
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<tr>
<td>Combined</td>
<td>258</td>
<td>218</td>
<td>33</td>
<td>7</td>
<td>54.9:1</td>
<td>2.49</td>
<td>0.1-0.5</td>
</tr>
</tbody>
</table>

*Includes plants with ratings similar to those of Fla. 7060 and the F₁: 0-2 for plants injected with strain 90-14 and 0-1 for plants injected with strain XV56(pXV9006) (0 = necrosis; 1 = 1-20%, and 2 = 21-40%).

*Includes classes with ratings different from those of parent or F₁ generations: 3-4 for plants injected with strain 90-14 and 2-4 for plants injected with strain XV56 containing pXV9006 (3 = 41-60% necrosis, and 4 = 61-80%).

Analysis was done at 40 h when all H7998 leaflets had reached a 5 in the necrosis rating scale, where 5 = 81-100% necrosis, but when leaflets of the susceptible genotype Fla. 7060 had no necrosis.

Analysis was done at 48 h when all H7998 leaflets had reached complete necrosis but when leaflets of the susceptible genotype Fla. 7060 had no necrosis (necrosis rating = 0).


discussion

Differentiation of the hypersensitive reaction from the susceptible reaction in plants is facilitated with most systems by the time difference for reactions. Rapid necrosis of tissue inoculated with high populations (10⁶ cfu/ml) of pathogenic bacteria is routinely used to identify the hypersensitive reaction (8). However, the difference in time for hypersensitive and susceptible reactions is not great for some systems, and it is difficult to differentiate the reactions (5,9). The hypersensitive reaction in H7998 inoculated with strain 90-14 of X. c. vesicatoria can be distinguished from the susceptible reaction, even though the hypersensitive reaction proceeds rather slowly after initiation. In the F₂ progeny of crosses of H7998 with the susceptible genotype, the hypersensitive reaction in some plants can be distinguished from a susceptible reaction by rapid necrosis. However, in other plants, necrosis occurs at intermediate times, and it is impossible to assess whether the reactions are hypersensitive or susceptible. In addition, development of necrosis in the inoculated area after initiation proceeded at different rates among the plants.

The possibility existed that variation in reactions among plants in the F₂ generation might be caused by environmental factors that were not controlled, although we attempted to standardize environmental factors that might affect development of the hypersensitive reaction. Therefore, the consistency of plant reactions was tested with asexually propagated plants that differed in development of necrosis. Electrolyte leakage from inoculated tissues, a more objective measure of necrosis than are visual ratings, was consistently negatively correlated with internal populations of the bacterium. In addition, the bacterial populations in leaves of the plants with different reactions were negatively correlated with timing of necrosis. This work corroborated earlier work with pepper, which showed a negative relationship between time to confluent necrosis after inoculation with high numbers of cells and the maximum population of bacteria that developed in leaves inoculated with low numbers of cells (5). The data supported the assumption that variations in reactions among populations of plants were not artifacts.

The hypersensitive reaction caused by X. c. vesicatoria in plants of H7998 was inherited by more than one gene, which is in agreement with Whalen et al (16). This was evident from the continuous distribution of plants in the F₂ population at a time when infiltrated areas of the resistant genotype were completely necrotic and those of the susceptible genotype had no necrosis. A model suggesting control of hypersensitivity by three genetic loci was presented. Necrosis was largely controlled by recessive gene action where the F₁ progeny had necrosis only slightly greater than the susceptible parent (Figs. 1A and 2A and B). It is important to note that bacterial populations in F₁ plants were not different from those in the susceptible parent at 48 h but were intermediate to both parents at later times. In previous field studies, resistant × susceptible F₁ plants were also intermediate to the parents (12,13). Thus, the necrosis reaction appears highly recessive, yet the resistance of the F₁ plants to X. c. vesicatoria appears more additive.

The three-gene model suggests only 1.5% of F₂ plants would breed true for rapid necrosis reaction. In a field study, approximately 7% of the F₂ plants were as resistant as H7998 (12). However, when F₁ progeny were grown out from five "resistant" F₁ plants the following year, only one bred true, which would fit well with the three-gene model. Further support for the theory that more than one gene is involved is found in the work of Whalen et al (14). On the basis of F₂ segregation with different rating criteria, they suggest two genes control resistance (hypersensitivity). The multigenic inheritance of hypersensitive reaction and the quantitative inheritance of resistance to bacterial spot under field conditions (12) superficially support the concept that the hypersensitive reaction is the mechanism for resistance under field conditions. However, other genetic factors may also
be involved. There was a significant correlation between hypersensitive reaction and field resistance in an F2 population, but the correlation coefficient was only 0.37 (14). Furthermore, resistant plants had either rapid or slow development of hypersensitive reactions, while plants with lower levels of resistance also had either rapid or slow development of hypersensitive responses. Further work is underway to help clarify this genetic system.

The multigenic inheritance of the hypersensitive reaction reported in this study is unusual among plant-microbe interactions and does not appear to fit the conventional gene-for-gene hypothesis. In the F2 progeny derived from crossing H7998 with the susceptible genotype, the inheritance of hypersensitivity was controlled by more than one gene in the plant. Only one avirulence gene, avrRdx, when inserted into a race 2 strain was responsible for a ratio (54:9:1) in an F2 population derived from H7998 × Fla. 7060 that was apparently similar to that of the wild-type race 1 strain. We are not aware of other documentation of this phenomenon.

Several models offer possible explanations, e.g., 1) the presence of several resistance genes in the host, which each interact with the avirulence gene in the pathogen at various levels of intensity; 2) the negative or positive modification of a single-gene resistance by several other genes in the host; and 3) the presence of two resistance systems in H7998, a multigenic system and a single-gene system. In the latter model, the interaction of the multigenic system with the pathogen would accelerate, or delay, the onset of necrosis associated with a single-gene resistance system. These models are being investigated.

De Feyter et al. (3) described a unique situation between cotton and X. c. malvacearum with avirulence genes in which they referred to two different types of gene-for-genes interactions. In the first, strains of X. c. malvacearum carrying avrB4 were avirulent in congenic cotton lines carrying either of two different resistance loci. In the second, one locus appeared to confer resistance in cotton against three avr genes. Crute (2) also refers to a number of exceptions to the gene-for-gene relationship.

Although the relationship between hypersensitivity and field resistance requires further study, we believe that the hypersensitive reaction could be used for screening progeny in a breeding program with H7998 as a source of resistance. Selection should be for the most rapid development of necrosis, similar to that of the resistant parent, because that reaction is associated with the lowest population of bacterial cells in the leaves after infection. Selection of such plants will require the screening of hundreds of plants, however, because the number of plants with rapid necrosis is low in a segregating population. Plants selected for rapid necrosis should be field tested under conditions favoring bacterial spot to verify their resistance levels.

LITERATURE CITED