Interaction of the *asc* Locus in F_8 Paired Lines of Tomato with *Alternaria alternata* f. sp. *lycopersici* and AAL-Toxin

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ABSTRACT

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Purified AAL-toxin and single conidial isolates of *Alternaria alternata* f. sp. *lycopersici* were used to analyze F_8 paired lines of tomato for their respective interaction with the *asc* locus. Reaction to the pathogen was inherited as a single locus dominant for resistance in a segregating F_9 population of 2,064 plants derived by selfing progeny tested F_8 asc^+/asc plants obtained by intercrossing F_7 asc^+/asc^+ and asc/asc full-sibs. Reaction to the AAL-toxin and the pathogen, assessed simultaneously on 390 F_9 progeny from a single F_8 asc^+/asc plant, showed no independent assortment for the two reactions. The parental ($asc^+$and *asc*), F_1 (asc^+/asc). F_9 (asc^+ and *asc*), and F_8 (asc^+/asc) plants revealed three significantly different levels of toxin sensitivity, which were inherited as an incomplete dominant and corresponded to the genotype at the *asc* locus.

Alternaria alternata (Fr.) Keissler f. sp. *lycopersici* causes Alternaria stem canker of tomato and produces host-specific pathotoxins (AAL-toxins, analogs TA and TB) in culture and in infected host tissue (10). Preliminary information based on segregation ratios in a limited F_2 population suggested that reaction to both the pathogen and crude culture filtrates of the pathogen containing AAL-toxins is controlled by a single gene with two alleles (*asc* locus) that exhibits complete dominance for pathogen resistance and incomplete dominance for toxin sensitivity (4). These data further suggest that if the *asc* locus controls both functions, the AAL-toxins function as pathogenicity factors (5,11) and would be a more stringent selection tool in applied plant breeding programs than the pathogen because they distinguish homozygous- and heterozygous-genotypes resistant to the pathogen. In addition, the *asc* locus would be suitable for physiological studies of the functional gene controlling the host-pathogen interaction using the AAL-toxin as a molecular probe if both reactions are controlled by the same locus.

Compatible or incompatible host-pathogen interactions are frequently mediated by single gene differences. However, differences in the physiological state of resistant and susceptible infected host plants often are due to spurious biochemical events, which result from allelic differences between closely linked, but non-disease-related, loci (3). In an attempt to circumvent this problem, Daly (2) used near-isogenic lines of wheat, previously developed by Loegering and Harmon (6), to evaluate the relationship of several physiological effects correlated with the resistance response in wheat to *Puccinia graminis* f. sp. *tritici*. Complementary studies with lines near-isogenic for *Sr*6 and *Sr*11 genes revealed that certain physiological effects (e.g., increased peroxidase and indoleacetic acid decarboxylation activities) were secondary phenomena and not primary determinants of disease resistance (2). To evaluate critically the function of putative resistance gene products, it is desirable to study alleles of the gene in question in a uniform genetic background.

The objectives of this study were to develop advanced generation genetic materials near-isogenic for the *asc* locus and to test the hypothesis that the alleles at the *asc* locus controlled both canker formation and differential sensitivity to the purified AAL-toxin TA. If the *asc* locus controls both interactions in the F_8 generation, the derived lines represent suitable genetic material for biochemical studies of the host-pathogen interactions using AAL-toxins to elucidate the metabolic dysfunction leading to symptom expression and establish the role of AAL-toxins as pathogenicity factors with potential use in fixing the dominant alleles in breeding lines.

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MATERIALS AND METHODS

Plant materials. Tomato (*Lycopersicon esculentum* Mill.) plants were grown in UC soil mix (7) in a heated greenhouse (23–29 C) with supplemental fluorescent and incandescent lighting (14 hr light, 10 hr dark). For progeny testing during near-isogenic line development, individual seeds were planted in 6-oz Styrofoam cups (punched with drainage holes) and packed in 35- × 50-cm metal flats (35 cups per flat). Plants retained for seed production were grown to maturity in 1- or 5-gal plastic containers and fertilized twice per month with Hoagland's solution.

Crosses were performed as described elsewhere (8). Fruits matured in 6–8 wk and contained 15–300 seeds per fruit. To harvest the seeds, the contents of each fruit were emptied into a beaker, covered with 1% HCl for 30 min and rinsed thoroughly with tap water in a plastic sieve. Seeds were dried overnight at 23 C before planting or storage. The starting material for development of the derived lines was the F_1 hybrid (asc^+/asc) from a cross between the cultivars ACE 55 VFN (asc^+) and Earlypak 7 (*asc*). The F_1 was selfed to produce a segregating F_2 population. The *asc*/*asc* genotypes were identified and eliminated by inoculating with *A. a.* f. sp. *lycopersici*. The heterozygous resistant (asc^+/asc) plants were distinguished from the homozygous resistant (asc^+) by the intermediate AAL-toxin sensitivity of asc^+/asc genotypes in the detached leaflet bioassay (described below). Ten asc^+/asc individuals were selected and selfed to produce the segregating F_3. The selection process was continued similarly through the F_6 generation, at which time a single, selfed fruit was taken from an F_6 heterozygous plant (designated F_6-85) and 35 seeds were grown out in individual 30-cm pots. The F_7 plants were inoculated with *A. a.* f. sp. *lycopersici* and selected resistant and susceptible plants were selfed for progeny testing. Plants identified as asc^+ (F_7-85-12, F_7-85-14) and *asc* (F_7-85-1, F_7-85-4, F_7-85-6) by progeny testing were selfed and intercrossed to obtain asc^+/asc^+, asc^+/asc, and *asc*/*asc* individuals in an F_7 background.

Fungal cultures. The single conidial isolate of *A. a.* f. sp. *lycopersici* (designated AS27-3) used for toxin accumulation was obtained from a naturally infected tomato plant in San Diego County, CA, as previously described (4). The stock isolate was maintained by periodic mass transfer on corn meal agar (CMA) and grown at 22–25 C under constant fluorescent illumination. Occasionally, susceptible tomatoes were inoculated with conidia of AS27-3 and the fungus was reisolated from resulting cankers to ensure continued pathogenicity of the cultures. Mature, sporulating cultures stored at 4 C in the dark retained pathogenicity for at least 1 yr. Inoculum was produced by aseptically transferring conidia from the parental culture to CMA slants, followed by incubation at 25 C for 7–14 days under constant fluorescent illumination.

Inoculation procedure. CMA slants with mature colonies of *A. a.* f. sp. *lycopersici* were flooded with about 5 ml of sterile water. Conidia, mycelium, and a small amount of agar were scraped into a mortar and mixed. The mixture was sufficiently viscous to adhere to a wounded tomato petiole or stem throughout the inoculation procedure. Two inoculation procedures were used. For genotypic classification of plants to be saved as parents for the next generation, two opposing leaflets per plant of 4- to 12-wk-old tomato plants were excised, the leaflets discarded, and inoculum applied to the leaflet scars. The entire leaf was then enclosed in a plastic bag, sealed with tape, and plants with bagged leaves placed under a bench for shading. The bags were removed after 48 hr, and inoculated leaves were rated for canker symptoms after an additional 48 hr on the greenhouse bench. Petioles of susceptible tomatoes darkened and collapsed at the inoculation site and brown streaks extended several centimeters on each side of the canker. After being rated, the entire leaf was removed and susceptible plants were grown to maturity without further evidence of Alternaria stem canker symptoms. For simple progeny tests, cotyledons of 3- to 4-wk-old tomato seedlings were excised with a scalpel and inoculum was applied as above. Flats were placed in a plastic inoculation chamber and misted at regular intervals for 48 hr. Plants were removed to the greenhouse bench and rated for canker development after 2 to 7 days.

TABLE 1. Progeny test of F_7 and parental genotypes of tomato for reaction to *Alternaria alternata* f. sp. *lycopersici*

Plant	Number inoculated[a]	Cankers formed	Genotype
ACE 55 VFN[b]	70	0	asc^+/asc^+
Earlypak 7[b]	70	70	*asc/asc*
ACE × Earlypak	70	0	asc^+/asc
F_6-85	35	7	asc^+/asc
F_7-85-1	35	35	*asc/asc*
F_7-85-4	35	35	*asc/asc*
F_7-85-5	35	35	*asc/asc*
F_7-85-6	35	34[c]	*asc/asc*
F_7-85-8	70	20	asc^+/asc
F_7-85-10	34	10	asc^+/asc
F_7-85-12	58	0	asc^+/asc^+
F_7-85-13	35	7	asc^+/asc
F_7-85-14	35	0	asc^+/asc^+
F_7-85-15	35	4	asc^+/asc
F_7-85-19	35	12	asc^+/asc
F_7-85-4 × Earlypak	35	35	*asc/asc*
F_7-85-6 × Earlypak	34	32[d]	*asc/asc*
F_7-85-1 × F_7-85-12	35	0	asc^+/asc
F_7-85-6 × F_7-85-12	37	0	asc^+/asc

[a] Inoculum of pathogen applied to leaf scars created by excising two opposing leaflets from 4- 12-wk-old tomato plants. Inoculated sites enclosed in plastic bags for 48 hr and rated for canker development after an additional 48 hr.

[b] ACE 55 VFN and Earlypak 7 are commercial tomato cultivars resistant and susceptible, respectively, to both field and culture-produced inoculum of *A. a.* f. sp. *lycopersici.*

[c] One plant died before rating.

[d] Two plants died before rating.

TABLE 2. Genotypic classification by F_9 progeny test of 109 F_8 tomato lines derived from F_7 plants heterozygous for the *asc* locus[a]

Parent	F_8 Genotype asc^+/asc^+	asc^+/asc	*asc/asc*	X^{2b}	Prob.
F_7-85-10	4	13	4	1.2	0.5
F_7-85-13	7	20	5	2.24	0.25
F_7-85-15	5	24	4	6.88	0.025
F_7-85-19	6	9	8	2.42	0.25
Total	22	66	21	4.87	0.5

[a] A minimum of 28 individual F_9 progeny from each of the 109 F_8 plants tested for reaction to *Alternaria alternata* f. sp. *lycopersici.* Inoculum applied to stem scars created by excising cotyledons from 3- to 4-wk-old tomato seedlings, and the plants held in a mist chamber for 48 hr, transferred to a greenhouse bench for an additional 2–7 days, then rated for canker development.

[b] Data tested for fit to a 1:2:1 ratio.

Toxin bioassay. AAL-Toxin (TA) was purified from cell-free culture filtrates of *A. a.* f. sp. *lycopersici* as previously described (9), except that solid-phase extraction on octyldecylsilane replaced butanol extraction and barium acetate precipitation (1). Results of toxin analysis by high-performance liquid chromatography and proton nuclear magnetic resonance, indicated that the TA used in this study was more than 99% pure (1). Analog TA was used exclusively in this study because it could be obtained pure in greater quantities than TB and is representative of the natural mixture because both analogs have the same host-specificity and specific activity (9). In general, three tomato leaflets were excised under water at an oblique angle and placed in a covered petri dish containing a 9-cm disk of Whatman No. 1 filter paper saturated with 3 ml of the appropriate toxin dilution. The petri plates were placed in airtight plastic boxes, both were arranged to minimize shading, and incubated in a growth chamber under constant illumination at 26 C for 48 hr. For the study of possible independent assortment of toxin sensitivity and pathogen susceptibility in the F_9 population, four leaflets were used per plate. Bioassays were rated on a scale from 0 to 4 after 48 hr with: 0 = no visible necrosis, 1 = 1–25% necrosis, 2 = 25–50% necrosis, 3 = 50–75% necrosis, 4 = 75–100% necrosis.

RESULTS

Progeny testing was used to confirm the genotypes of all plants used as parents from the F_6 generation forward (Table 1). The fit of F_8 progeny (obtained by selfing F_7 asc^+/asc individuals) to a $1asc^+/asc^+$:$2asc^+/asc$:$1asc/asc$ ratio was examined by genetically classifying 109 F_8 plants using progeny testing. A minimum of 28 F_9 individuals from each of the 109 selfed F_8's were inoculated with *A. a.* f. sp. *lycopersici.* Plants giving 100% susceptible progeny were *asc/asc*, those giving zero out of 28 were asc^+/asc^+, and those giving at least one canker (but less than 100%) were asc^+/asc. A ratio of $22asc^+/asc^+$:$66asc^+/asc$:$21asc/asc$ ratio was obtained that fit the proposed ratio at the 0.05 probability level (Table 2). The 66 F_8 asc^+/asc plants (Table 2) produced a segregating population of 2,064 F_9 individuals in which 511 cankered plants were recorded (3.04:1 ratio of resistant to susceptible plants). The data fit the model of a single dominant gene with two alleles for resistance to Alternaria stem canker at the 0.975 probability level.

To investigate possible independent assortment of the genetic control of host reaction to the pathogen and host sensitivity to AAL-toxins, a cross between F_7-85-4 (*asc/asc*) and F_7-85-12 (asc^+/asc^+) was made. The F_1 heterozygote was selfed and 390 F_2 progeny of the sister F_7 lines were grown for 4 wk under greenhouse conditions. Each plant was individually labelled and two leaflets from each plant were removed for detached leaflet bioassay. The plants were then inoculated with *A. a.* f. sp. *lycopersici* and evaluated for canker development as described above. Of the 99 plants that developed cankers, all were sensitive (toxin severity index of 3 or above) to AAL-toxin (TA) at 2.6 μg/ml. All of the remaining 291 canker-free plants had toxin severity indicies <3 when tested at 2.6 μg/ml TA.

To further correlate toxin sensitivity with the allelic state of the

TABLE 3. Correlation of sensitivity to AAL-toxin and susceptibility to *Alternaria alternata* f. sp. *lycopersici* in parental and derived lines of tomato

Generation	Genotype	Cankers[a]	Mean TSI[b]
P_0 (Early Pak 7)	*asc*/*asc*	35	3.97 a
F_9-85-6	*asc*/*asc*	35	3.97 a
F_1 (Early Pak/ACE 55)	asc^+/*asc*	0	2.20 b
F_7-85-7 × F_7-85-12	asc^+/*asc*	0	2.10 b
P_0 (ACE 55 VFN)	asc^+/asc^+	0	0.68 c
F_9-85-12	asc^+/asc^+	0	0.81 c

[a] A total of 35 plants of each genotype inoculated. Inoculum applied to stem scars created by excising cotyledons from 3- to 4-wk-old tomato seedlings, and the plants held in a mist chamber for 48 hr, transferred to a greenhouse bench for an additional 2–7 days, then rated for canker development.

[b] TSI = Toxin Severity Index: 0 = no necrosis of leaflets, 1 = 1–25%, 2 = 25–50%, 3 = 50–75%, 4 = >75%. A total of 70 leaves of each genotype evaluated by the standard detached leaf bioassay. Means followed by the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test. df = 414, MSE = 0.311732.

asc locus, 35 plants from each of the parents (ACE and Earlypak 7), the F_1 progeny (asc^+/asc), the F_8 heterozygote (F_7 85-6 × F_7 85-12), and the near-isogenic F_9 homozygotes (F_9 85-6 and F_9 85-12), were tested for canker and toxin sensitivity as above. An analysis of variance of the results followed by Duncan's multiple range test indicated three significantly different levels of toxin sensitivity that corresponded to the genotype at the *asc* locus (Table 3).

DISCUSSION

The results of the genetic analysis of the *asc* locus presented here are consistent with the hypothesis that resistance to *A. a.* f. sp. *lycopersici* and the AAL-toxins is governed by a single gene with two alleles. Resistance to the pathogen shows complete dominance, whereas sensitivity to the toxin exhibits incomplete dominance. The data from over 2,000 inoculated plants fit the expected 3:1 ratio (resistant:susceptible). Also, an analysis of 109 F_8 individuals, whose genotypes were determined by progeny testing each plant, fit a 1:2:1 ratio at the 5% significance level. No evidence of independent assortment between the toxin sensitivity and pathogen reaction loci was obtained in thousands of inoculations and leaflet bioassays from the F_1 through the F_6 or in the full-sib F_7 to F_9 families reported here. Analysis of variance showed significant differences between the mean toxin severity index of the three genotypes with the heterozygote showing intermediate sensitivity.

The resistant and susceptible F_8 paired lines developed here (e.g., 85-6 and 85-12) can now be increased by self-fertilization under greenhouse and field conditions to provide large quantities of uniform genetic material, but differing at the *asc* locus, available for detailed investigations of the biochemical basis of disease resistance in this system. The data also confirm that the purified AAL-toxins are selective chemical probes for the *asc* locus and can be used to detect this locus in segregating populations in lieu of the pathogen. The AAL-toxins also offer a more stringent selection tool than the pathogen in breeding programs where fixation of the asc^+/asc^+ genotype is desired.

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