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Tomato resistance to *Alternaria* stem canker: localization in host genotypes and functional expression compared to non-host resistance

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Summary. The *Alternaria* stem canker resistance locus (*Asc*-locus), involved in resistance to the fungal pathogen *Alternaria alternata* f. sp. *lycopersici* and in insensitivity to host-specific toxins (AAL-toxins) produced by the pathogen, was genetically mapped on the tomato genome. Susceptibility and resistance were assayed by testing a segregating F_2 population for sensitivity to AAL-toxins in leaf bioassays. Linkage was observed to phenotypic markers solanifolium and sunny, both on chromosome 3. For the *Asc*-locus, a distance of 18 centiMorgan to solanifolium was calculated, corresponding to position 93 on chromosome 3. This map position of the resistance locus turned out to be the same in three different resistant tomato accessions, one Dutch and two American, that are at least 40 years apart. AAL-toxin sensitivity in susceptible and resistant tomato genotypes was compared with AAL-toxin sensitivity in a non-host *Nicotiana tabacum* during different levels of plant cell development. In susceptible and resistant tomato genotypes, inhibitory effects were demonstrated at all levels, except for leaves of resistant genotypes. However, during pollen and root development, inhibitory effects on susceptible genotypes were larger than on resistant genotypes. In the non-host *Nicotiana tabacum*, hardly any effects of AAL-toxins were demonstrated. Apparently, a cellular target site is present in tomato, but not in *Nicotiana tabacum*. It was concluded that three levels of AAL-toxin sensitivity exist: (1) a susceptible host sensitivity, (2) a resistant host sensitivity, (3) a non-host sensitivity, and that the resistance mechanism operating in tomato is different from that operating in *Nicotiana tabacum*.

Key words: *Alternaria alternata* f. sp. *lycopersici* – Disease resistance – Host-specific toxins – Tomato resistance – Non-host resistance

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Introduction

Alternaria stem canker in tomato is caused by the fungal pathogen *Alternaria alternata* f. sp. *lycopersici* and is characterized by formation of dark-brown cankers on stems and necrosis of leaf tissue between the veins (Grogan et al. 1975). The fungus produces toxins, the AAL-toxins, that have been demonstrated to play a major role in pathogenesis (Gilchrist and Grogan 1976; Siler and Gilchrist 1983). These toxins exhibit the same host-specificity as the pathogen itself: tomato genotypes susceptible to the fungus are sensitive to AAL-toxins and genotypes resistant to the fungus are insensitive to AAL-toxins. Insensitivity to AAL-toxins has been demonstrated for several other species of the *Solanaceae* (e.g. *Solanum tuberosum, Nicotiana tabacum*) and species of eight other families (Gilchrist and Grogan 1976). A locus called *Asc* is involved in resistance and insensitivity to AAL-toxins in tomato (Clouse and Gilchrist 1987). Genetic studies on near-isogenic tomato lines different at the *Asc*-locus have shown that pathogen resistance is inherited as a single gene expressing complete dominance, while insensitivity to purified AAL-toxins is controlled by a gene expressing incomplete dominance. Homozygous susceptible lines are 1,000 × more sensitive to AAL-toxins than homozygous resistant lines, while their F_1 hybrid has an intermediate sensitivity (Clouse and Gilchrist 1987).

Cellular targets are demonstrated for a number of toxins: T-toxin of *Helminthosporium maydis* affects mitochondria (Dixon et al. 1982); tentoxin produced by *Alternaria tenuis* affects the chloroplast (Steele et al. 1976) and the bacterial toxins; tabtoxin, produced by *Pseudomonas tabaci* (Turner 1981, 1986) and phaseolotoxin, produced by *Pseudomonas phaseolicola* (Mitchell 1976) inhibit biosynthetic pathways. Tabtoxin was shown to

inhibit glutamine synthetase (GS) (Turner 1981, 1986), while phaseolotoxin inhibits ornithine carbamoyl transferase (OCTase) (Ferguson and Johnston 1980). Inhibition of GS and OCTase, both involved in nucleotide biosynthesis, causes chlorosis in leaves (Gilchrist 1983).

In contrast to host-specific toxins from other formae speciales of *Alternaria alternata,* AAL-toxins do not induce rapid loss of electrolytes from sensitive tissues (Kohmoto et al. 1982). In electron microscopic studies, an effect of AAL-toxins on mitochondria of toxin-treated leaves was observed (Park et al. 1981). Gilchrist (1983) suggested that AAL-toxins act upon aspartate carbamoyl transferase (ACTase), a key enzyme in pyrimidine biosynthesis. Recently it has been found that, in several plant species, ACTase is located in chloroplasts (Shibita et al. 1986, 1987; Doremus and Jagendorf 1985).

Effects of AAL-toxins in tomato and functioning of tomato resistance on cellular and higher levels of plant cell development were described in Witsenboer et al. (1988). At all levels, sensitivity to AAL-toxins was demonstrated in susceptible and resistant genotypes. For protoplasts, suspension cells and calli, no differences in sensitivity to AAL-toxins could be demonstrated between susceptible and resistant tomato genotypes. Tomato insensitivity to AAL-toxins could thus not be recognized at the cellular level with the used genotypes and in the applied experimental system.

At higher levels of plant cell development; i.e. leaves, roots and pollen, differences in sensitivity to AAL-toxins were observed (Witsenboer et al. 1988; Bino et al. 1988). Effects on different resistant genotypes were comparable, however, until now it was not known whether the same resistance locus is involved in different tomato genotypes.

The effects of AAL-toxins on a non-host genotype were studied only in pollen, and three levels of sensitivity were found: for non-hosts complete insensitivity to AAL-toxins was observed at toxin concentrations that inhibited resistant tomato pollen, while pollen of susceptible genotypes were more sensitive to AAL-toxins than pollen of resistant genotypes (Bino et al. 1988).

In this paper, we describe: (1) the genetic mapping of the *Asc*-locus in three different resistant tomato genotypes, and (2) non-host resistance of *Nicotiana tabacum* compared to tomato resistance by studying effects of AAL-toxins at cellular and higher levels of plant cell development of hosts and non-hosts.

Materials and methods

Plant material

Lycopersicon esculentum VF 11, LA 1182 (sunny [sy], solanifolium [sf] {3}; albescent [alb], multifurcata [mua] {1}), LA 1164 (variabilis [var], notabilis [not] {7}; anthocyaninless Hoffman's [ah], marmorata [marm] {9}) and LA 291 (anthocyaninless [a], hairless [hl] {11}), obtained from C. Rick (Tomato Genetics Stock Centre, Department of Vegetable Crops, University of California, Davis and Msk 9 (Koornneef et al. 1987) were used as susceptible genotypes. *L. esculentum* LA 1166 (clausa [clau], divergens [di] {4}; incana [icn], anthocyanin gainer [ag] {10}), LA 1444 (white virescent [wv], dwarf [d] {2}; anthocyanin free [af], trifoliate [tf] {5}), LA 1665 (diageotropica [dgt] {1}; lutescent [1], anthocyanin looser [al] {8}), obtained from C. Rick (see above), cultivars Floramerica (obtained from Petoseed Co., Saticoy, California) and Moneymaker (obtained from IVT 86363, Wageningen, The Netherlands) were used as resistant genotypes. *Nicotiana tabacum* cv Petit Havana SR1 (Maliga et al. 1973) was used as non-host genotype.

In vivo plant material was grown in a greenhouse at 25 °C, 60% relative humidity. In vitro plants were grown sterile in glass containers with MS salts (Murashige and Skoog 1962) and 2% sucrose in a growth chamber: 15 h photoperiod, 1,500 lx, 25 °C. In vitro-grown plants were used for: protoplast isolation, root induction on excised shoots and shoot and callus induction on leaf discs. Roots were induced on MS salts, 2% sucrose. Experiments on root induction were performed using six shoots at each applied AAL-toxin concentration; after 14 days, root lengths were measured. Callus was induced and maintained on MS salts, 3% sucrose, 2 mg/l naphthalene acetic acid, 1 mg/l 6-benzylamino purine. For callus growth, 16 calli with a total weight of 0.5 g were placed on agar plates in quadruplicate; after 3 weeks, callus growth on each plate was determined. Shoots were induced on MS salts, 3% sucrose, 1 mg/l zeatine, 0.1 mg/l indole acetic acid. Shoot induction on leaf discs was carried out in triplicate with 8 discs per petri dish.

Isolation of *Nicotiana tabacum* SR-1 protoplasts was carried out as described by Negrutiu et al. (1987), with media based on that of Kao and Michayluk (1975) and the agarose bead-type system (Shillito et al. 1983). Out of 1.10^6 *Nicotiana tabacum* SR1 protoplasts plated without selective pressure, 40% developed into minicalli within a period of 3 weeks.

Effects of toxins were analysed 3 weeks after exposing the protoplasts to AAL-toxins (1) by counting minicalli and determining the relative plating efficiencies, and (2) by measuring diameters of minicalli under the microscope with a measuring ocular.

Purification and characterization of AAL-toxins. Purification and characterization of AAL-toxins was performed as described in Witsenboer et al. (1988).

Leaf bioassay

Leaflets of 4-week-old greenhouse-grown plants were excised and placed in plastic petri dishes on 9-cm discs of filter paper saturated with 3 ml of 0.1 μM AAL-toxins in H_2O and 3 ml of H_2O for the controls (Gilchrist and Grogan 1976). The petri dishes were sealed with Nescofilm and incubated in a growth chamber 22°–25 °C, 1,500 lx. Bioassays were carried out in triplicate. Symptoms were analysed after a period of 3 days. A sensitive response for AAL-toxins implies that more than 50% of the leaf surface exhibited necrosis; an insensitive toxin response implies that less than 10% of the leaf surface exhibited necrosis.

Linkage and recombination percentages. Linkage and recombination percentages were analysed with computer program Linkage-1, version 3.50 for Apple Macintosh (Suiter et al. 1987). Recombination percentages were corrected with Haldane's mapping function for determination of distances from markers (Haldane 1919).

Table 1. Segregation data for AAL-toxins insensitivity locus (*Asc; asc* = recessive allele) in combination with all tested markers

Marker	Position[a]	N[b]	Segregation[c]				χ^{2}[d]
			X, *Asc*	X, *asc*	x, *Asc*	x, *asc*	
dgt	1L152	89	36	18	28	7	1.87
wv	2L 41	389	249	64	56	20	1.24
d	2L 70	389	225	70	80	14	3.29
sy	3L 46	389	225	59	60	25	2.78
sf	3S111	389	274	21	31	63	151.08
clau	4S 0	99	77	2	18	2	2.30
di	4L 89	99	73	4	22	0	1.19
af	5L 14	389	190	54	115	30	0.11
tf	5L 40	389	222	60	83	24	0.06
yv	6L 34	108	69	8	24	7	2.75
c	6L104	108	70	12	23	3	0.16
var	7S 0	103	66	18	13	6	0.89
not	7L 40	103	63	21	16	3	0.74
l	8S 0	89	56	17	8	8	4.64
al	8L 67	89	50	16	14	9	1.87
ah	9L 24	103	60	19	19	5	0.11
marm	9L 62	103	62	21	17	3	0.96
ag	10L132	99	70	3	25	1	0.00
hl	11L 48	108	58	22	27	1	7.09
alb	12L 0	389	252	68	53	16	0.13
mua	12L 45	389	255	76	50	8	2.45

[a] L = long arm; S = short arm
[b] N = number of individuals in segregating F_2 population
[c] x = marker gene (mutant allele); X = wildtype allele of mutant gene
[d] $p < 0.001$ if $\chi^2 > 10.83$

Results

Mapping of the Asc-locus in resistant tomato lines

Crosses were made between susceptible and resistant tomato marker lines in such a way that seedling markers were present on each chromosome and 80% of the genome was genetically covered. After selfing of F_1 plants, at least 100 individuals of each F_2 population were scored for marker segregation and leaves were tested in a leaf bioassay for AAL-toxin sensitivity. Segregation data and χ^2 values are shown in Table 1. The *Alternaria* stem canker resistance locus turned out to be linked to solanifolium (sf) and distantly linked to sunny (sy; $p < 0.10$ if $\chi^2 > 2.71$) on chromosome 3. No linkage was observed to any other marker ($p < 0.001$ if $\chi^2 > 10.83$). A recombination percentage of $15\% \pm 5\%$ was observed between *Asc* and sf in LA 1444. This corresponds to a distance of 17.8 ± 5 map units from sf. The *Asc*-locus was mapped at position 93 on chromosome. 3. Two other resistant genotypes, the American cultivar Floramerica and the Dutch cultivar Moneymaker, were tested for linkage of the *Asc*-locus to solanifolium and sunny. Data are summarized in Table 2. Linkage to sf was again observed. The *Asc*-locus was mapped at position 100 ± 10 in Floramerica and at position 90 ± 10 in Moneymaker, leading to the conclusion that the map position of the *Asc*-locus is the same, at least in these three tomato cultivars.

Effects of AAL-toxins on root growth, shoot induction on leaf discs and callus growth of resistant tomato versus non-host Nicotiana tabacum

Shoots of resistant Moneymaker and non-host *Nicotiana tabacum* were placed on root induction medium with AAL-toxins. AAL-toxins inhibited root growth of Moneymaker and *Nicotiana tabacum* at all tested concentrations. In Table 3, lengths of roots after 2 weeks on root induction medium are shown. At toxin concentrations of 3 μM, root growth of Moneymaker was relatively more reduced than root growth of *Nicotiana tabacum* (root length was 16% of the controls for Moneymaker and 30% for *Nicotiana tabacum*) and at 15 μM AAL-toxins, root growth of Moneymaker was 1% of the control, while root growth of *Nicotiana tabacum* was only inhibited to 20% of the control.

Leaf discs of Moneymaker and *Nicotiana tabacum* were analysed for shoot induction. Results, scored after 6 weeks, are summarized in Table 4. For Moneymaker, no shoots were observed at toxin concentrations of 0.3 μM and higher, for *Nicotiana tabacum* shoots were formed at all tested toxin concentrations and shoot for-

Table 2. Segregation data of AAL-toxins insensitivity locus (*Asc; asc* = recessive allele) in combination with solanifolium (sf) and sunny (sy) for LA 1444, Floramerica and Moneymaker

Line	Marker[a]	N[b]	Segregation[c]				χ^{2}[d]	Recombination (%)	Distance from x
			X, *Asc*	X, *asc*	x, *Asc*	x, *asc*			
LA 1444	sy	389	225	59	60	25	2.78	0.43+0.04	
	sf	389	274	21	31	63	151.0	0.15+0.05	17.8
Floramerica	sy	105	63	16	19	7	0.51	0.48+0.08	
	sf	105	80	7	2	16	56.98	0.10+0.10	11.2
Moneymaker	sy	100	59	19	19	3	1.15	0.41+0.08	
	sf	100	65	3	13	19	38.31	0.17+0.10	20.8

[a] sy = sunny; sf = solanifolium
[b] N = number of individuals in segregating F_2 population
[c] x = marker gene (mutant allele); X = wildtype allele of mutant gene
[d] $p<0.001$ if $\chi^2>10.83$; $p<0.10$ if $\chi^2>2.71$

Table 3. Effects of AAL-toxins (0, 0.6, 3.0, 15.0 μ*M*) on root growth of resistant Moneymaker (Resistant) and *Nicotiana tabacum* (non-host)

AAL in μ*M*	Moneymaker (R)		*Nicotiana tabacum* (non-host)	
	$\bar{x}$ (mm)	%	$\bar{x}$ (mm)	%
0	71	100	36	100
0.6	51	72	16	45
3.0	11	16	11	30
15.0	0.4	1	7	20

Table 4. Effects of AAL-toxins (0, 0.06, 0.3, 1.5, 3.0, 7.5 μ*M*) on number of leaf discs of Moneymaker (Resistant) developing shoots and *Nicotiana tabacum* (non-host) in relative percentages

AAL in μ*M*	Moneymaker (R) (%)	*N. tabacum* (non-host) (%)
0	100	100
0.06	121	100
0.3	71	109
1.5	0	105
3.0	0	82
7.5	0	68

Table 5. Relative callus growth of Floramerica (Resistant) and *Nicotiana tabacum* (non-host) at increasing concentrations of AAL-toxins scored after 21 days

AAL in μ*M*	Floramerica (R)	*N. tabacum* (non-host)
0	100	100
0.6	52	92
3	34	109
15	40	99

Table 6. Relative plating efficiencies calculated as mini-calli (PE) and relative diameters (∅) of non-host *Nicotiana tabacum* in percentages of the control. Toxins were applied either at day 1 (A) or at day 15 (B)

AAL in μ*M*	A (t=1)		B (t=15)	
	PE (%)	∅ (%)	PE (%)	∅ (%)
0	100	100	100	100
0.3	95	89	98	79
1.2	84	85	86	71
3.0	81	81	87	71
12.0	76	67	86	67

mation was not inhibited up to toxin concentrations of 1.5 μ*M*. At a concentration of 7.5 μ*M* AAL-toxins, shoot induction on tobacco leaf explants was reduced to 70% of the controls. Effects of AAL-toxins on callus growth are summarized in Table 5. Fifteen micromolar AAL-toxins that inhibited resistant Floramerica to 40% of the control had no effect on callus growth of *Nicotiana tabacum*.

Effects of AAL-toxins on protoplasts and minicalli of Nicotiana tabacum

Immediately following protoplast isolation, AAL-toxins were added to protoplast cultures and 3 weeks later, effects on number and growth of minicalli were determined. Results are summarized in Table 6 A. The number of minicalli was reduced to 76% of the controls and the diameter of minicalli decreased to 67% of the controls, at the highest tested toxin concentrations of 12 μ*M*.

Fifteen days after protoplast isolation, when protoplasts had developed into minicalli of approximately 16 cells, AAL-toxins were added and again effects were scored after 3 weeks. Results are summarized in Table 6 B. The number of minicalli decreased to 86% of the

controls and the diameter of minicalli was inhibited to 67% of the controls at AAL-toxin concentrations of 12 μM.

Discussion

Genetic mapping of the tomato resistance locus

In this paper, we report the genetic mapping of the *Asc*-locus in three different resistant tomato genotypes. For the map position, we calculated position 93 on chromosome 3. Moneymaker is a Dutch inbred line, available since 1946; Floramerica is an American cultivar developed in the 1960s and LA 1444 was also developed in the United States (C. Rick, Tomato Genetics Stock Centre, Department of Vegetable Crops, University of California, Davis). Our conclusion is that one *Asc*-locus is involved in tomato resistance to *Alternaria* stem canker as demonstrated for these three tomato genotypes.

Tomato resistance compared to non-host resistance

AAL-toxin effects were studied on roots, leaf discs and calli of resistant tomato and *Nicotiana tabacum*, and protoplasts and minicalli of *Nicotiana tabacum*. Effects on pollen and leaves of *Nicotiana tabacum* and effects on susceptible and resistant tomato were described earlier (Gilchrist and Grogan 1976; Witsenboer et al. 1988; Bino et al. 1988). Results are summarized in Table 7. For susceptible and resistant tomato, inhibition by AAL-toxins was demonstrated at all tested levels (Table 7, Witsenboer et al. 1988). Results are summarized in Table 7. For susceptible and resistant tomato, inhibition by AAL-toxins was demonstrated at all tested levels (Table 7, Witsenboer et al. 1988). Apparently a cellular target site is present in tomato. On non-host *Nicotiana tabacum*, hardly any inhibitory effects were demonstrated (Table 7), so it is quite possible that no cellular target site is present in tobacco. Witsenboer et al. (1988) reported that differences in sensitivity to AAL-toxins between susceptible and resistant tomato genotypes were observed at the pollen, leaf and root level, but not during shoot induction from leaf discs and at the callus and protoplast level (Table 7). It was concluded that resistance in tomato might be related to a higher level of plant cell differentiation. Because in *Nicotiana tabacum* no effects of AAL-toxins were demonstrated except for roots, differences in sensitivity to AAL-toxins between resistant tomato and non-host *Nicotiana tabacum* were observed at all levels, except for leaves (Table 7). Thus, resistance in tobacco is independent of differentiation level.

Our conclusions are (1) that three levels of sensitivity to AAL-toxins exist: a susceptible host sensitivity, a resistant host sensitivity and a non-host sensitivity; and (2) that at the levels described here, in *Nicotiana tabacum* a resistance mechanism operates different from that in resistant tomato, which provides a higher level of insensitivity to AAL-toxins.

Until now, non-host resistance is generally described as reaction of a plant to a certain pathogen, and observations are hard to quantify (Heath 1980). It is obvious that differences in resistance mechanisms to a certain pathogen exist even within one host. For instance, in the *Cladosporium fulvum* – tomato interaction, where resistance implies a hypersensitive reaction, it can be easily discriminated from immunity. Immunity results in death of only a few cells and is even a quicker response than the resistant reaction, where cell death leads to necrotic lesions. Here differences in mechanisms should be present (Lazarovits and Higgins 1976a, b). Differences in resistance mechanisms were also shown by Elliston et al. (1977) for the *Colletotrichum* – bean interaction. In this interaction, differences in resistance mechanisms could be discriminated by heat treatment of plants: two resis-

Table 7. Summary of AAL-toxins effects on susceptible (S) and resistant (R) tomato genotypes and tobacco (non-h)

	Effects AAL-toxins[a]			Differences in sensitivity[b]	
	Host		Non-host	S/R	Non-h/R
	Susceptible tomato	Resistant tomato	tobacco		
Pollen	++	+	−	+	+
Leaves	++	−	−	+	−
Roots	+++	++	+	+	+
Shoots	++	+	−	−	+
Callus	++	+	−	−	+
Protoplasts	++	+	−	−	+

[a] +++/++/+: inhibitory effects in order of decreasing effect, +++ relative to ++ and + indicate quantitative differences in effect within one line, compared to the effect on the susceptible tomato genotype; −: no inhibitory effects

[b] +: differences in sensitivity; −: no differences in sensitivity

tance responses, mature tissue resistance and systemic protection were reduced by heat treatment of plants. Heat treatment did not diminish the effectiveness of race-specific resistance and induced local protection.

For the *Alternaria alternata* f. sp. *lycopersici*–plant interaction, no symptoms were observed on resistant host and non-host plants (Grogan et al. 1975). By studying effects of AAL-toxins at different levels of plant cell development of host and non-host plants, qualitative and quantitative differences in resistance mechanisms could be investigated. Whether specificity of AAL-toxins in tomato is due to their target site(s) or to specific resistance mechanisms is the subject of further investigations.

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