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Inheritance and genetic mapping of resistance to *Alternaria alternata* f. sp. *lycopersici* in *Lycopersicon pennellii*

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Abstract The fungal pathogen *Alternaria alternata* f. sp. *lycopersici* produces AAL-toxins that function as chemical determinants of the Alternaria stem canker disease in the tomato (*Lycopersicon esculentum*). In resistant cultivars, the disease is controlled by the *Asc* locus on chromosome 3. Our aim was to characterize novel sources of resistance to the fungus and of insensitivity to the host-selective AAL-toxins. To that end, the degree of sensitivity of wild tomato species to AAL-toxins was analyzed. Of all members of the genus *Lycopersicon*, only *L. cheesmanii* was revealed to be sensitive to AAL-toxins and susceptible to fungal infection. Besides moderately insensitive responses from some species, *L. pennellii* and *L. peruvianum* were shown to be highly insensitive to AAL-toxins as well as resistant to the pathogen. Genetic analyses showed that high insensitivity to AAL-toxins from *L. pennellii* is inherited in tomato as a single complete dominant locus. This is in contrast to the incomplete dominance of insensitivity to AAL-toxins of *L. esculentum*. Subsequent classical genetics, RFLP mapping and allelic testing indicated that high insensitivity to AAL-toxins from *L. pennellii* is conferred by a new allele of the *Asc* locus.

Key words AAL-toxins · Alternaria stem canker · *Asc* locus · *Lycopersicon esculentum* · RFLP linkage analysis

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Introduction

Wild germplasm provides a rich supply of valuable traits for the cultivated tomato (*Lycopersicon esculentum* Mill.), such as resistance to diseases and stress conditions (Taylor 1986). In the search for new sources of resistance to pathogens, accessions within the genus *Lycopersicon* have been screened, and subsequently, chromosomal fragments were introgressed by backcross breeding (Doolittle 1953; Alexander and Hoover 1955). Most of the resistances were inherited in a simple manner, often conferred by a single dominant locus (Stevens and Rick 1986). With respect to the fungus *Alternaria alternata* f. sp. *lycopersici* (Fries) no such locus was (intentionally) introgressed from wild species since resistance was already present in some tomato cultivars (Grogan et al. 1975). In order to characterize novel resistance factors for this pathogen, wild tomato relatives can be used as a potential source of new resistance alleles.

The facultative saprophytic fungus *A. alternata* f. sp. *lycopersici* produces AAL-toxins that are involved in the pathogenesis of the Alternaria stem canker disease in susceptible tomato cultivars (Gilchrist and Grogan 1976; reviewed by Van der Biezen et al. 1994a). Characteristic of the disease is the formation of dark-brown cankers on stems and necrosis of leaf tissue. The host range of the fungus and its host-selective AAL-toxins is assumed to be restricted to tomato cultivars, where the disease is controlled by the *Asc* locus (Clouse and Gilchrist 1987). *Asc* has been mapped on chromosome 3 (Witsenboer et al. 1989) and is expressed as a completely dominant locus for resistance to pathogen infection. However, insensitivity to AAL-toxins is regulated by *Asc* as an incompletely dominant trait. The AAL-toxins have been purified from fungal cultures (Siler and Gilchrist 1982; Clouse et al. 1985), and have been structurally characterized (Caldas et al. 1994).

The development of the Alternaria stem canker disease is based upon the relative sensitivity of the host to the AAL-toxins. Therefore, these toxins have been used

to investigate sensitivity responses of several tissues of tomato and other plant species (Siler and Gilchrist 1983, Clouse and Gilchrist 1987; Bino et al. 1988; Fuson and Pratt 1988; Witsenboer et al. 1988; 1989; 1992; Moussatos et al. 1993; Lamprecht et al. 1994). Three different responses to AAL-toxins were reported: (1) a sensitivity response from susceptible (*asc/asc*) tomato cultivars, (2) a moderate insensitivity response from resistant (*Asc/Asc*) tomato cultivars, and (3) a high insensitivity response from other plant species (in the literature often referred to as non-hosts). Harmful effects of the AAL-toxins were demonstrated on all tested tomato tissues with all *Asc* genotypes. However, the concentration of toxins necessary to cause symptom development varied for different tissues, indicating differences in toxicity between tissues; e.g. relatively high AAL-toxin concentrations are needed to cause inhibition of pollen tube growth while low levels are sufficient to cause necrosis in leaf tissue.

Our objective was to characterize resistance to the fungus *A. alternata* f. sp. *lycopersici* and insensitivity to its host-selective AAL-toxins of wild tomato species. Therefore, AAL-toxins were used to determine the sensitivity responses of leaves and roots of members of the genus *Lycopersicon*. *L. cheesmanii* Riley was revealed to be sensitive to AAL-toxins, while a high degree of insensitivity was observed in *L. pennellii* Corr. and *L. peruvianum* (L.) Mill. Genetic analyses demonstrated that high insensitivity to AAL-toxins from *L. pennellii* is inherited as a single complete dominant locus in tomato. This *high insensitivity* locus has been placed on the tomato restriction fragment length polymorphism (RFLP) map and was shown to be allelic to the *Asc* locus of *L. esculentum* cultivars.

Materials and methods

Plant materials

Seeds of *L. chilense* (G1.1707), *L. chmielewskii* (CPRO 731089), *L. glandulosum* (G1.568), *L. hirsutum* (G1.1708), *L. parviflorum* (G1. 1604), *L. pennellii* (LA716, G1.1611), *L. peruvianum* (G1.1860), *L. pimpinellifolium* (G1.1704), *Capsicum frutescens* (G2.300), *C. annuum* (G2.478), *L. esculentum* cultivar Allround (G1.837) and F_2 seeds (CPRO 81175) derived from a cross between Allround×*L. pennellii* were obtained from P. Lindhout, CPRO-DLO, Wageningen, The Netherlands. Near-isogenic lines (NILs) of *L. esculentum* differing with respect to the *Asc* locus (referred to as isolines) were derived from tomato cultivars Ace 55 VFN (resistant, *Asc/Asc*) and Earlypak 7 (susceptible, *asc/asc*) by continuous selfing (F_9) of heterozygotes and were obtained from D. Gilchrist, University of California, Davis, USA (Clouse and Gilchrist 1987). Seeds from 13 ecotypes of *Arabidopsis thaliana* were provided by the *Arabidopsis* Seed Stock Center, Nottingham, UK. Seeds of *Solanum tuberosum* (Hudson×Mansour) were obtained from *Ropta ZPC*, Metslawier, The Netherlands. Seeds of *Petunia hybrida* (R18), *Nicotiana tabacum* (Petit Havanna SR1), *Brassica napus* (Westar), *Zea mays* and *Antirrhinum majus* were obtained from our own collection. The tomato cultivar Sonatine was supplied by *De Ruiter Seeds*, Bleiswijk, The Netherlands and Moneymaker was obtained from K. Theres, Universität zu Köln, Germany. The *A. alternata* f. sp. *lycopersici* susceptible (*asc/asc*) Tester-3 (*L. esculentum*) that contains the phenotypic seedling markers *sy* (sunny: cotyledons bleached, leaves emerge yellow), *bls* (baby lea syndrome: hypocotyl anthocyanin lacking), and *sf* (solanifolia: leaves entire) was selected from an F_2 between LA1004 and LA1182 (obtained from C. Rick, Tomato Genetics Stock Center, University of California Davis, USA).

Genetic crosses

All controlled crosses were performed in the greenhouse using standard emasculation and pollination techniques. The interspecific hybrid was made by crossing a susceptible *L. esculentum* (*asc/asc*) line (Clouse and Gilchrist 1987) with *L. pennellii* as the staminate parent. The F_2 progenies were obtained by self-pollinating the F_1 plants. The BC_1 population was derived by backcrossing the F_1 with *L. esculentum* (*asc/asc*) as the pistillate parent. Heterozygotes (*Asc/asc*) were made by reciprocal crosses between the susceptible (*asc/asc*) and resistant (*Asc/Asc*) isolines; the F_1 progeny was verified for heterozygosity by segregation analysis of *Asc* in the F_2 by seedling and leaflet assays. Crosses between the Tester-3 line (*sy/sy*, *bls/bls*, *asc/asc*, *sf/sf*) and Moneymaker (*Asc/Asc*), Sonatine (*Asc/Asc*), and *L. pennellii* were made with the latter as the staminate parent. Plants were grown under standard greenhouse conditions at 25°C and 60% relative humidity.

Tests for fungal resistance and susceptibility

Inoculations were carried out by spraying plants that were grown until the 2–3 true leaves stage in the greenhouse with *A. alternata* f. sp. *lycopersici* spores (10^6 spores/ml), followed by incubation for 72 h at 100% humidity at 24°C. Observations were made 1 week later when similarly inoculated susceptible (*asc/asc*) control plants had developed Alternaria stem canker symptoms.

Purification of AAL-toxins

Cell-free culture filtrates of *A. alternata* f. sp. *lycopersici* were obtained as described by Gilchrist and Grogan (1976). Purification of AAL-toxins was carried out according to Clouse et al. (1985). Characterization of AAL-toxins was done following Witsenboer et al. (1988).

Seedling and leaf bioassays

Seedlings and leaflets were assayed for sensitivity to AAL-toxins according to essentially identical procedures. A Whatman filter paper was saturated with 3 ml of the appropriate AAL-toxins dilution in a 9 cm diameter petri dish. After placement of approximately 30 seeds or 3–5 leaflets from greenhouse-grown plants (2–3 true leaves stage) on the filter, the dish was sealed with Nescofilm and left in a climate cabin under standard conditions (16 h, 3000 lx, 25°C). As the rate of development and severity of symptoms is a function of the AAL-toxin concentration, the bioassays were terminated after a defined period to avoid differences in severity of symptoms as a function of time (Gilchrist and Grogan 1976). The development of leaf necrosis, starting between the veins and eventually spreading through the whole tissue, was scored after 3 days. Toxin severity ratings were determined visually by assessing the percentage of the leaflet lamina area with necrotic symptoms and is expressed as the Leaf Sensitivity Index (LSI) with 0=no visible necrosis, 1=1–25% necrosis, 2=26–50% necrosis, 3=51–75% necrosis, and 4=76–100% necrosis (Clouse and Gilchrist 1987). Leaf bioassays were carried out in triplicate. The inhibition of root growth was determined in seedling assays after 5 days by measurement of the root length relative to that of seedlings germinated on water. The percentage of root growth inhibition in seedling assays was rated on a scale from 0 to 4 using the Root Sensitivity Index (RSI) with: 0=no inhibition, 1=1–25% inhibition, 2=26–50% inhibition, 3=51–75% inhibition, 4=76–100% inhibition. Seedling assays were performed in duplicate.

Recombinant DNA technology

Recombinant DNA work was performed using standard procedures (Sambrook et al. 1989).

Plant DNA isolation

Leaf tissue (4 g) was ground in liquid nitrogen using a pestle and mortar. After addition of 3 ml 50°C lysis buffer [10% (v/v) 7X (Flow Laboratories), 50 mM EDTA, 2% (w/v) Sarkosyl, 0.75 M phenol pH 8, 7 M urea], the mixture was shaken. Subsequently, 4 ml phenol/chloroform pH 8 was added and, following vigorous mixing, the solution was centrifuged. The DNA was precipitated from the aqueous phase with 3 M NaAc and isopropanol, washed with 70% (v/v) ethanol, dried and dissolved in 1 ml TE (10 mM Tris-HCl, 1 mM EDTA pH 8) with 20 μg RNAase at 37°C. After additional phenol/chloroform extractions the DNA was again precipitated, washed, dried and dissolved in 1 ml TE.

DNA blot hybridizations

10 μg of total DNA was digested with restriction endonucleases (Boehringer) and the resulting fragments were size-fractionated by electrophoresis through 0.7% (w/v) agarose gels. After depurination, denaturation and neutralization, the DNA was transferred to Hybond-N^+ membranes by vacuum blotting (LKB) for 1 h using 10xSSC (1.5 M NaCl, 0.15 M Sodiumcitrate), and fixed to the membrane by 0.4 M NaOH treatment according to the supplier (Amersham). Hybridizations were carried out in 1% (w/v) BSA, 1 mM EDTA, 0.5 M $NaHPO_4$, pH 7.2, 7% SDS (Church and Gilbert 1984) at 60°C for 16 h in a Hybaid hybridization oven, using [^{32}P]dCTP (Amersham) radiolabeled probes prepared by random priming of gel-purified DNA. Following hybridization the filters were washed down to 2xSSC, 0.1% (w/v) SDS or to 0.2xSSC, 0.1% (w/v) SDS at 60°C and exposed to preflashed Kodak X-omat AR films at –70°C with intensifying screens. Filters were re-used several times after removing the previous probe by boiling for 2 min in 0.5% (w/v) SDS.

RFLP mapping

Filters containing DNA of AAL-toxin sensitive and insensitive F_2 and BC_1 plants, digested with enzymes that show an RFLP between the *L. esculentum* and *L. pennellii* parents, were hybridized with the following radiolabeled RFLP markers: *Cab*11 (Schwartz et al. 1991); CT85, CT248, TG42, TG134, TG152, TG284, TG359, TG377 and TG442 (Tanksley et al. 1992); ST233 (Rommens et al. 1992); ACT1 (Overduin et al. 1993); and SLJ1515T (Thomas et al. 1994). Recombination values and map positions were calculated using the computer package JOINMAP (version 1.3) with a critical LOD score of 3.0 for linkage and 0.05 for mapping using Kosambi's mapping function (Stam 1993).

Results

Sensitivity of *Lycopersicon* species to AAL-toxins

To characterize resistance in tomato to the fungus *A. alternata* f. sp. *lycopersici*, responses of wild tomato species to the host-selective AAL-toxins were determined. In addition to the *Lycopersicon* species, other Solanaceae and accessions of some more families were tested for their sensitivity to AAL-toxins. The relative levels of sensitivity to AAL-toxins, of on average of 20 individuals tested per species, were investigated by quantifying the development of necrosis in detached leaf assays (1.0 μM AAL-toxins), and by determination of the inhibition of root growth in seedling assays (0.2 μM AAL-toxins). For classification, the Sensitivity Index (SI) was used (see Materials and methods), which is expressed as percentage of leaf necrosis (LSI) or as percentage of inhibition of root growth (RSI). As a reference for the classification of sensitivity to AAL-toxins, *L. esculentum* near-isogenic lines (*Asc* isolines) were used (Clouse and Gilchrist, 1987). The concentrations of AAL-toxins were chosen such that discrimination of the three genotypes was possible. At these levels, the AAL-toxins had a little effect on leaves and roots of the resistant (*Asc/Asc*) plants (RSI 1/LSI 1). However, maximum root growth inhibition (RSI 4) and leaf necrosis (LSI 4) were observed on susceptible (*asc/asc*) genotypes, while intermediate responses (RSI 3/LSI 3) were obtained from the heterozygous (*Asc/asc*) control plants (Table 1).

Table 1 Sensitivity to AAL-toxins of *Lycopersicon* species, other Solanaceae and members of other plant families

		Sensitivity Index[a]	
		Roots (RSI)	Leaves (LSI)
L. esculentum[b]	*Asc/Asc*	1	1
	Asc/asc	3	3
	asc/asc	4	4
L. cheesmanii		4	4
L. chilense		2	2
L. chmielewskii		1	1
L. glandulosum		2	2
L. hirsutum		1	1
L. parviflorum		1	1
L. pennellii		0	0
L. peruvianum		0	0
L. pimpinellifolium		1	1
Capsicum annuum		0	0
C. frutescens		0	0
Nicotiana tabacum		0	0
Petunia hybrida		0	0
Solanum tuberosum		1	0
Antirrhinum majus		0	0
Arabidopsis thaliana		1	0
Brassica napus		1	0
Zea mays		0	0

[a] Sensitivity to AAL-toxins expressed as the percentage of root growth inhibition and percentage of leaf necrosis monitored in seedling assays (0.2 μM) and leaf assays (1.0 μM), numerically rated using the Leaf (LSI) and Root Sensitivity Index (RSI) with 0=no symptoms, 1=1–25%, 2=26–50%, 3=51–75%, 4=76–100% symptoms

[b] Near-isogenic lines (Clouse and Gilchrist 1987)

The wild tomato species showed distinct sensitivity responses; a strong sensitivity response (LSI 4/RSI 4) was observed for *L. cheesmanii*, some moderate insensitivity responses in, e.g. *L. pimpinellifolium* (LSI 1/RSI 1) and *L. chilense* (LSI 2/RSI 2), and high insensitivity responses (LSI 0/RSI 0) in *L. peruvianum* and *L. pennellii*. Almost no responses (LSI 0/RSI 0), indicating high insensitivity, could be detected on plant species outside the genus *Lycopersicon*. Minor inhibition of root growth

Table 2 Sensitivity to AAL-toxins expressed as the Leaf Sensitivity Index (LSI)[a] of *L. esculentum Asc* genotypes[b], *L. cheesmanii*, *L. pennellii* and F_1, F_2 and BC_1 progenies of the interspecific cross *L. esculentum* (*asc/asc*)×*L. pennellii*

AAL-toxins (μm)	*L. esculentum*[b]			*L. cheesmanii*	*L. pennellii*	*L. esculentum* (*asc/asc*)×*L. pennellii*		
	Asc/Asc	*Asc/asc*	*asc/asc*			F_1	F_2[c]	BC_1[c]
0.0	0	0	0	0	0	0	0 0	0 0
0.02	0	0	1	1	0	0	0 1	0 1
0.1	0	0	3	3	0	0	0 3	0 3
0.2	0	1	4	4	0	0	0 4	0 4
1.0	1	3	4	4	0	0	0 4	0 4
10	3	4	4	4	0	0	0 4	0 4
40	4	4	4	4	0	0	0 4	0 4
60	4	4	4	4	1	1	1 4	1 4
100	4	4	4	4	1	1	1 4	1 4

[a] Sensitivity to AAL-toxins was monitored by leaf assays (20 plants/class, 3–5 leaflets/dish in triplicate) and classified according to the percentages of necrosis development numerically rated using the Leaf Sensitivity Index as follows: 0=no necrosis, 1=1–25%, 2=26–50%, 3=51–75%, 4=76–100% necrosis

[b] Near-isogenic lines (Clouse and Gilchrist 1987)

[c] Population consisting of two classes with different responses to AAL-toxins

(RSI 1) was observed in some species. Significant mutual differences in sensitivity responses within a species were not observed. The host-selective character of the AAL-toxins was confirmed by fungal infection tests; in contrast to *L. pennellii,* which was highly insensitive to AAL-toxins and resistant to infection with a spore suspension of *A. alternata* f. sp. *lycopersici*, the *L. cheesmanii* accession was sensitive to the toxins and susceptible to fungal infection. The development of the symptoms of the Alternaria stem canker disease of *L. cheesmanii* was identical to that of the susceptible (*asc/asc*) *L. esculentum* isoline. In addition, leaf assays with different AAL-toxin concentrations showed that *L. cheesmanii* is as sensitive to AAL-toxins as the *asc* isoline (Table 2).

Sensitivity of progenies from *L. esculentum* (*asc/asc*)×*L. pennellii* crosses to AAL-toxins

To study the high insensitivity response from *L. pennellii* to AAL-toxins, this species was crossed to susceptible (*asc/asc*) *L. esculentum* plants. The resulting interspecific hybrids were used to produce F_2 and backcross (BC_1) populations. Sensitivity responses were determined as degree of necrosis development in leaf assays (LSI) using different AAL-toxin concentrations (Table 2). The ratings expressed in the LSI reflect a mean of all data obtained by leaf assays containing 3–5 leaflets per plant (performed in triplicate) from, on average, 20 plants per genotype. Although slight variation in the degree of necrosis was observed, the results were sufficiently clear to allow classification of the responses using the Sensitivity Index. Necrotic lesions (LSI 1) were observed in leaves of the susceptible (*asc/asc*) isoline at 0.02 μM AAL-toxins (sensitivity response). Leaves from the resistant (*Asc/Asc*) isoline showed similar levels of necrosis at 1.0 μM AAL-toxins (moderate insensitivity response). As a result of incomplete dominance, comparable sensitivity responses were detected from the heterozygous (*Asc/asc*) genotype at 0.2 μM AAL-toxins (intermediate sensitivity response). All three *Asc* genotypes developed severe leaf necrosis (LSI 4) at higher AAL-toxin levels.

The *L. pennellii* parent and its progenies were revealed to be highly insensitive to AAL-toxins (LSI 0). However, insensitivity was not absolute since some necrosis (LSI 1) could be detected at higher AAL-toxin levels (60 μM), although symptoms were not exacerbated at increased concentrations (LSI 1 at 100 μM). As a result of genetic segregation in the F_2 and BC_1 progenies (see next section), two sensitivity responses to AAL-toxins were recognized: (1) high insensitivity derived from the *L. pennellii* parent, and (2) sensitivity inherited from the susceptible (*asc/asc*) *L. esculentum* isoline. In contrast to the resistant (*Asc/Asc*) tomato cultivars (*Asc* isoline, Allround, Moneymaker, Sonatine) that show necrosis at relatively low AAL-toxin levels (1.0 μM), *L. pennellii* and progenies from the interspecific hybrid display comparable symptoms only at high AAL-toxin concentrations (60–100 μM). Differences in sensitivity responses to several AAL-toxin concentrations from the expected homozygous and heterozygous *high insensitivity* genotypes could not be detected within the F_2 population (Tables 2, 3). Moreover, the high insensitivity response in the F_2 appeared to be identical to that of the heterozygous genotypes of the interspecific F_1 and BC_1 progenies. Therefore, under the conditions described here, it is concluded that high insensitivity from *L. pennellii* is regulated as a completely dominant trait. This is in contrast to the incomplete dominance of insensitivity to AAL-toxins expressed by heterozygous *L. esculentum* (*Asc/asc*) genotypes. High insensitivity from *L. pennellii* to AAL-toxins is maintained in F_1, F_2 and BC_1 progenies of the interspecific hybrid. Inoculation of 75 F_2 plants with spores of *A. alternata* f. sp. *lycopersici* showed that resistance and susceptibility to the fungus was correlated with insensitivity and sensitivity to AAL-toxins, respectively (Table 3).

Table 3 Segregation of resistance to infection with *Alternaria alternata* f. sp. *lycopersici* spores, insensitivity to AAL-toxins, and of the morphological markers sunny (*sy*), baby lea syndrome (*bls*) and solanifolia (*sf*) in F_2 and BC_1 progenies of *L. esculentum* (*asc/asc*)×*L. pennellii* and intraspecific controls

Population		Total	Sensitivity to AAL-toxins		Morphological markers					
			Insensitive	Sensitive	+	*sy*	+	*bls*	+	*sf*
Isoline×*L. pennellii*	F_2[a]	75	60	15 (20%)	–		–		–	
	F_2	1180	980	200 (17%)[b]	–		–		–	
	BC_1	80	47	33 (41%)	–		–		–	
Tester-3×*L. pennellii*	F_2	217	190	27 (12%)[b]	209	8 (4%)[b]	186	31 (14%)[b]	169	48 (22%)
Tester-3×Sonatine	F_2	288	173	55 (24%)	172	56 (25%)	169	59 (26%)	175	53 (23%)
Tester-3×Moneymaker	F_2	282	210	72 (26%)	208	74 (26%)	210	72 (26%)	213	69 (24%)
Allround×*L. pennellii*	F_2	407	407	0 (0%)	–		–		–	
Allround×Moneymaker	F_2	122	122	0 (0%)	–		–		–	

[a] Infection with *A. alternata* f. sp. *lycopersici* spores
[b] Deviation from expected ratios (F_2 3:1, BC_1 1:1) significant at $P<0.005$

Genetic segregation of high insensitivity to AAL-toxins from *L. pennellii*

The inheritance of resistance to *A. alternata* f. sp. *lycopersici* and high insensitivity to AAL-toxins was analyzed in F_2 and BC_1 progenies of the interspecific hybrid derived from the susceptible (*asc/asc*) isoline and *L. pennellii*. Also, a chromosome 3 marker line (Tester-3) was crossed with *L. pennellii* to produce an interspecific F_2 progeny for simultaneous analysis of the inheritance of high insensitivity to AAL-toxins and three morphological markers (*sy*, *bls*, *sf*; see Materials and methods) in an *L. esculentum/L. pennellii* hybrid background. As control, the segregation of insensitivity to AAL-toxins and these phenotypic markers was determined in two intraspecific F_2 populations derived from the same Tester-3 line and two *L. esculentum* parents, i.e. Moneymaker and Sonatine. Both tomato cultivars are resistant to *A. alternata* f. sp. *lycopersici* and insensitivity to AAL-toxins is comparable to that of the resistant (*Asc/Asc*) isoline (data not shown). Moneymaker is an obsolete introgression-free cultivar, while Sonatine is a modern breeding line carrying several introgressed resistance loci (Van der Beek et al. 1992). In addition, Sonatine contains at least two chromosomal regions that are not associated with resistance to any pathogen. These regions may either be remnants of chromosomal segments of wild tomato relatives, resulting from (incomplete) back crossing, or may encode traits that pertain to the breeding selection criteria and, consequently, may have been unconsciously selected for. Interestingly, one of the polymorphic regions maps to the predicted position of the *Asc* locus on an integrated linkage map of chromosome 3 (Koornneef et al. 1993). In this study we analyzed whether the introgressed region spans the *Asc* locus and, therefore, might be a new source of resistance to *A. alternata* f. sp. *lycopersici* (see next section). For segregation analysis, F_2 plants were tested for resistance to infection with fungal spores, and for insensitivity to various concentrations of AAL-toxins in leaf assays (Table 3).

In total, 1472 F_2 and 80 BC_1 plants from the interspecific hybrids were assayed for resistance to fungal infection and/or insensitivity to AAL-toxins (Table 3). In contrast to the intraspecific crosses (*L. esculentum*×*L. esculentum*), the progenies of the interspecific crosses (*L. esculentum*×*L. pennellii*) showed segregation distortion for sensitivity to AAL-toxins and for the phenotypic markers *sy* and *bls*. In all interspecific populations, skewed ratios were observed in favor of *high insensitivity* derived from *L. pennellii*. With respect to monogenic segregation ratios, the observed reduction in the number of progenies (*asc* isoline×*L. pennellii*) that were susceptible to fungal infection and/or sensitive to AAL-toxins is 31% in the F_2 ($n=1255$). In the BC_1 population an 18% reduction was observed in the number of AAL-toxin sensitive plants; however, due to the small population size ($n=80$) this deviation is not statistically significant. Concomitantly with a 52% decrease of AAL-toxin sensitive F_2 plants from the Tester-3×*L. pennellii* cross ($n=217$), a reduction in the number of plants carrying morphological markers was observed: 85% for *sy* and 43% for *bls*. It is concluded that the preferential transmission of *L. pennellii* alleles is not exclusively associated with resistance to *A. alternata* f. sp. *lycopersici* or insensitivity to AAL-toxins but is observed for *sy* and *bls* as well. Progenies of intraspecific crosses segregated in monogenic segregation ratios for all loci. Possibly, the low representation of plants with the alleles from the *L. esculentum* parents is related to the smaller fraction of viable seeds obtained from interspecific hybrids: 63% ($n=3110$) compared to 94% ($n=632$) of those from intraspecific control crosses.

Linkage analyses and allelism test of the *high insensitivity* and *Asc* loci

The *Asc* locus from several tomato cultivars has been shown to be genetically linked to *sf* on the classical linkage map (Witsenboer et al. 1989). Since *sf* is the most distal marker on the long arm of chromosome 3 it was presumed that the position of *Asc* is proximal to *sf*. Our classical linkage analyses (with intraspecific populations) experimentally justified this assumption: *Asc* was

Table 4 Segregation and recombination values of chromosome segments defined by the morphological markers sunny (*sy*), baby lea syndrome (*bls*) and solanifolia (*sf*), and sensitivity to AAL-toxins (*asc*) in interspecific and intraspecific F_2 progenies

Population		Total	Interval		Phenotypes[a]				χ^2 [b]	Recombination
			M_1	M_2	M_1M_2	M_1m_2	m_1M_2	m_1m_2		(%)[c]
Tester-3×*L. pennellii*	F_2	217	*sy*	*bls*	184	25	2	6	79.4	19.0±4.6
			bls	*asc*	172	14	18	13	51.2	20.1±4.2
			asc	*sf*	162	28	7	20	48.0	21.1±4.0
Tester-3×Sonatine	F_2	228	*sy*	*bls*	142	30	27	29	27.5	29.1±3.9
			bls	*asc*	150	19	23	36	61.1	20.5±3.3
			asc	*sf*	154	19	21	34	44.2	18.0±3.3
Tester-3×Moneymaker	F_2	282	*sy*	*bls*	178	30	32	42	52.6	25.4±3.3
			bls	*asc*	185	25	25	47	80.6	20.2±2.9
			asc	*sf*	188	22	25	47	84.9	17.9±2.8

[a] *M* wild type, *m* mutant
[b] If $\chi^2>11.3$ than $P<0.01$
[c] Recombination percentages with standard error calculated using JOINMAP

revealed to be linked to *sf* as well as to *bls*, in the order *bls* - *Asc* - *sf* (Table 4, Fig. 2). Despite the skewed segregation ratios in the interspecific progenies, recombination frequencies between all three phenotypic loci (*sy*, *bls*, *sf*), and insensitivity to AAL-toxins are of the same order of magnitude as for intraspecific progenies (Table 4). Between *sy* and *bls* a lower recombination fraction was found relative to that in intraspecific crosses. From the classical linkage analyses it is concluded that the locus from *L. pennellii* conferring resistance to *A. alternata* f. sp. *lycopersici* and high insensitivity to AAL-toxins is localized in the middle of the long arm of chromosome of 3. The position of this *high insensitivity* locus corresponds to the position of the *Asc* locus, suggesting that these loci are allelic.

To test the hypothesis that the locus conferring high insensitivity to AAL-toxins from *L. pennellii* is allelic to the *Asc* locus from *L. esculentum*, a test for allelism was employed. F_2 seeds from the interspecific hybrid derived from a resistant (*Asc/Asc*) tomato cultivar (Allround) and *L. pennellii* were tested for sensitivity to AAL-toxins in seedling and leaf assays. Among 407 F_2 progeny plants no recombinants were found that showed a sensitivity response to AAL-toxins in seedling or leaf assays, confirming that the two loci are very closely linked (<0.25 cM), and can therefore be are considered alleles (Table 3). It is concluded that *L. pennellii* carries an *Asc* allele conferring high insensitivity to AAL-toxins. We propose the symbols $Asc1^1$ for the allele from *L. esculentum* and $Asc1^2$ for the allele from *L. pennellii*. In contrast to $Asc1^1$ which confers insensitivity to *low* AAL-toxin concentrations (1.0 μM), $Asc1^2$ confers insensitivity to *high* AAL-toxin levels (100 μM).

RFLP linkage analysis of the *Asc* locus

To position the *Asc* locus on the tomato RFLP map, DNA of insensitive and sensitive F_2 and BC_1 individuals (*asc* isoline×*L. pennellii*) was digested with the appropriate enzymes and subjected to Southern hybridization analysis with chromosome 3-specific DNA markers (Fig. 1). For RFLP mapping a two-step procedure was applied. First, an approximate position of *Asc* was established using several RFLP markers on the long arm of chromosome 3 (Tanksley et al. 1992). To that end, DNA of 68 F_2 individuals (30 insensitive, 38 sensitive to AAL-toxins) and 33 BC_1 plants (16 insensitive, 17 sensitive to AAL-toxins) was analyzed with the following seven RFLP markers: TG42, TG152, TG284, TG359, TG377, ST233 and SLJ1515T. All markers were shown to be (distantly) linked to the *Asc* locus. The position of the *Asc* locus could not be separated from that of TG359. The chromosome 11-specific RFLP marker, ACT1, was shown not to be linked to high insensitivity to AAL-toxins (Overduin et al. 1993). To map the position of *Asc* with higher resolution, a larger number of plants of known genotype with respect to *Asc* needed to be screened with additional RFLP markers for recombination breakpoints in the region of the *Asc* locus. Because of the completely dominant nature of this locus, the precise *Asc* genotype of the AAL-toxin insensitive plants is not known. To avoid progeny testing, only plants that were sensitive to AAL-toxins (*asc/asc*) were used for RFLP analysis. A group of 140 AAL-toxin sensitive plants were first analyzed for

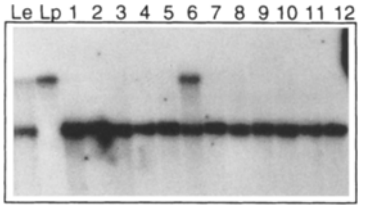

Fig. 1 Autoradiograph of a filter containing *Eco*RI-digested DNA of *Lycopersicon esculentum* (*asc/asc*) (*Le*), *L. pennellii* (*Lp*) and 12 interspecific F_2 individuals, hybridized to radiolabeled RFLP marker TG134. All 12 F_2 plants are sensitive to AAL-toxins (*asc/asc*) and, therefore, show the restriction fragment characteristic of the *L. esculentum* chromosome. Plant 6 also shows the restriction fragment characteristic of *L. pennellii* and hence contains one chromosome 3 that has undergone recombination in the interval between *asc* and TG134

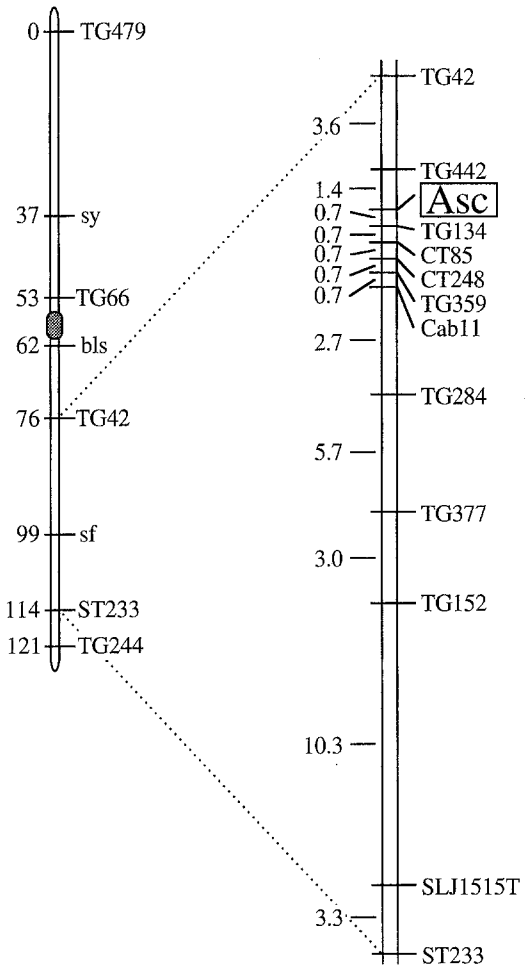

Fig. 2 Integrated linkage map of tomato chromosome 3 (left, Van der Biezen et al. 1994b) and a segment of chromosome 3 (right) containing the *Asc* locus constructed by RFLP linkage analysis. The *grey rectangle* denotes the centromere, positions (left) and distances (right) are expressed in centiMorgan (cM)

recombinations between TG42 and TG377 that flank the *Asc* locus. The 40 plants that showed recombinations in the TG42/TG377 interval (17 cM) were subsequently analyzed with RFLP markers that map within this chromosome segment: TG442, TG134, CT85, CT248, TG359 and *Cab*11. Mapping of the recombination breakpoints positioned the *Asc* locus between TG442 (1.4%±0.7% recombination) and TG134 (0.7%±0.5% recombination). The results of the RFLP analysis did not indicate the presence of more than one locus involved in resistance to *A. alternata* f. sp. *lycopersici* and insensitivity to AAL-toxins. To construct an RFLP linkage map the data of all F_2 (n=208) and BC_1 (n=33) plants with all 12 RFLP markers were used to calculate recombination values and map positions by the JOINMAP software (Fig. 2).

The tomato cultivar Sonatine contains an introgressed chromosomal fragment that extends at least from the position of TG284 to TG359 (Van der Beek et al. 1992). Leaf assays revealed that Sonatine is as sensitive to AAL-toxins as the resistant (*Asc*/*Asc*) isoline (data not shown). Genetic linkage analysis showed no evidence for distorted segregation (Table 3) and confirmed that insensitivity to AAL-toxins is conferred by the *Asc* locus (Table 4). The possibility that the introgressed segment spans the *Asc* locus was tested using an approach identical to that of Van der Beek et al. (1992). Southern blots containing DNA digested with *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Taq*I, or *Xba*I were hybridized to closely linked RFLP markers. The presence of introgressed DNA in Sonatine could be inferred if at least one of these enzymes showed an RFLP relative to the introgression-free cultivar, Moneymaker. First, the authenticy of the segment was confirmed with *Cab*11 (RFLP with *Eco*RV and *Hin*dIII), which maps between TG284 and TG359. Hybridization with CT248, CT85 and TG134 did not show RFLPs, indicating that the size of the introgression is not larger than the 5 cM determined by Van der Beek et al. (1992), and that it does not span the *Asc* locus.

Discussion

To study further the interaction between the fungus *A. alternata* f. sp. *lycopersici* and tomato (*L. esculentum*) our aim was to characterize new sources of resistance to the pathogen and insensitivity to its host-selective AAL-toxins. Wild tomato relatives (*Lycopersicon* spp.) were chosen for investigation because of the relative evolutionary proximity of the cultivated tomato and most wild species and the sexual compatibility between them. The host-selective AAL-toxins were used to screen one accession per species of the genus *Lycopersicon* for sensitivity reactions. Among several distinct insensitivity responses from some species, the *L. cheesmanii* accession was shown to be highly sensitive to AAL-toxins and susceptible to infection with *A. alternata* f. sp. *lycopersici*. It was previously found that only 25% of 265 tested tomato cultivars were susceptible to fungal infection; additional plant species from 16 genera, representing nine families, were not affected by the pathogen nor the AAL-toxins (Gilchrist and Grogan 1976). It is concluded that, in addition to some tomato cultivars, the host-range of *A. alternata* f. sp. *lycopersici* can be extended to *L. cheesmanii*.

L. pennellii and *L. peruvianum* appeared to be highly insensitive to AAL-toxins and did not show the typical Alternaria stem canker symptoms after infection with the fungal spores. *L. pennellii* can be sexually crossed to tomato and, therefore, this wild tomato species was chosen for further investigation. Genetic analyses showed that high insensitivity to AAL-toxins from *L. pennellii* is inherited as a single completely dominant locus in tomato. Segregation ratios of most loci in interspecific hybrids deviated from monogenic ratios, as is frequently observed in such crosses. In identical crosses between *L. esculentum* lines and *L. pennellii*, similarly skewed ratios were observed for morphological, isozyme and RFLP markers on most chromosomes (Rick 1969; Chetelat and DeVerna 1991; Eshed et al. 1992; Foolad and Jones 1993). Sensitivity to AAL-toxins, and the phenotypic markers *sf* and *bls* are clearly expressed in interspecific

progenies and, therefore, can be scored unambiguously. However, poor penetrance of *sy* in the presence of *L. pennellii* alleles may account for the low number of plants carrying this marker. Linkage analysis in interspecific progeny did not indicate suppression of recombination between markers flanking the *high insensitivity* locus (*bls* and *sf*). Suppression of recombination is usually observed in *L. esculentum*×*L. pennellii* crosses, albeit predominantly in centric regions (Rick 1969; De Vicente and Tanksley 1991; Tanksley et al. 1992).

Classical and RFLP genetic mapping using chromosome 3-specific markers and allelic testing indicated that the *high insensitivity* locus from *L. pennellii* is an allele of the *Asc* locus. We suggest the symbols $Asc1^1$ for the allele of *L. esculentum* and $Asc1^2$ for the allele of *L. pennellii*. By combining the linkage data of this study with that of others, an integrated genetic linkage map of tomato chromosome 3 was constructed (Van der Biezen et al. 1994b). It can be concluded that the three sensitivity responses of tomato to AAL-toxins are all conferred by different alleles of the *Asc* locus: (1) the sensitivity response of cultivars with the *asc/asc* genotype; (2) the moderate insensitivity response of cultivars with the $Asc1^1$ allele; and (3) the high insensitivity response conferred by $Asc1^2$ from *L. pennellii*. Some leaf necrosis (1–25%) was observed at high AAL-toxin concentrations (60–100 μM, Table 2) in the third class, while this was not detected for species outside the genus *Lycopersicon*, e.g. *N. tabacum* (data not shown). Similar observations were made when the degree of inhibition of pollen germination in the presence of AAL-toxins was determined (Bino et al. 1988). In these experiments, pollen of *Lycopersicon* species was shown to be less insensitive to AAL-toxins than that of non-*Lycopersicon* species, e.g. *N. tabacum*. These independent observations might indicate that the origin of insensitivity to AAL-toxins in species within *Lycopersicon* differs from that of species outside this genus.

Many disease resistance loci have been positioned on the tomato map (Tanksley et al. 1992). Most of these loci were introgressed from wild species. However, resistance to some pathogens (like *A. alternata* f. sp. *lycopersici*) was already present in tomato cultivars, e.g. moderate resistance to races of *Fusarium oxysporum* f. sp. *lycopersici* causing Fusarium wilt (Bohn and Tucker 1939). Higher levels of resistance to race 1 and race 2 of this fungal pathogen are conferred by loci from *L. pimpinellifolium*: the *I* locus for resistance to race 1 (Paddock 1950) and the *I-2* locus for resistance to race 2 (Laterrot 1976; Sarfatti et al. 1989). Additionally, three monogenic resistance loci were found in tomato derived from *L. pennellii*, conferring resistance to *F. oxysporum* races 1, 2 and 3 (McGrath et al. 1987; Scott and Jones 1989; Bournival et al. 1989, 1990; Sarfatti et al. 1991). These closely linked resistance loci do not map at the position of the traditional *I* and *I-2* loci from *L. pimpinellifolium* on chromosome 11, but were localized on chromosome 7. In contrast to the different resistance loci from *L. pimpinellifolium* and *L. pennellii* that confer resistances to the same *F. oxysporum* race, we report here that resistances to *A. alternata* f. sp. *lycopersici*, derived from *L. esculentum* ($Asc1^1$) and *L. pennellii* ($Asc1^2$) map to the same locus. Conversely, it has been found in tomato that one (complex) resistance locus can confer resistance to different pathogen races, e.g. loci derived from *L. esculentum*, *L. hirsutum* and *L. pimpinellifolium* confer resistance to several races of the fungus *Cladosporium fulvum*: *Cf-1*, *Cf-4* and *Cf-9* on chromosome 1, and *Cf-2* and *Cf-5* on chromosome 6 (Jones et al. 1993).

In tomato, *L. pennellii* has been a source of resistance to *F. oxysporum* (McGrath et al. 1987; Bournival et al. 1990) and an origin of susceptibility to *Xanthomonas campestris* (Wang et al. 1994) and *C. fulvum* (Hammond-Kosack and Jones 1994). This study indicates that the $Asc1^2$ allele from *L. pennellii* can be introgressed into tomato cultivars by RFLP-assisted breeding. Tomato lines carrying $Asc1^2$ can be useful when higher levels of insensitivity to AAL-toxins are in demand, e.g. when *A. alternata* f. sp. *lycopersici* overcomes the $Asc1^1$ cultivar resistance. Presently, we are using the $Asc1^2$ allele for position-based cloning of this locus (Martin et al. 1993). Yeast artificial chromosome (YAC) libraries with tomato genomic DNA have been screened for clones containing TG442 and TG134. To position the inserts relative to the *Asc* locus, plants are used with recombinations between *Asc* and one of the RFLP markers. A preselection is made for plants with recombinations in this region using the flanking phenotypical markers *bls* and *sf*.

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