Most AAL Toxin-Sensitive *Nicotiana* Species are Resistant to the Tomato Fungal Pathogen *Alternaria alternata* f. sp. *lycopersici*

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The phytopathogenic fungus *Alternaria alternata* f. sp. *lycopersici* produces AAL toxins required to colonize susceptible tomato (*Lycopersicon esculentum*) plants. AAL toxins and fumonisins of the unrelated fungus *Fusarium moniliforme* are sphinganine-analog mycotoxins (SAMs), which are toxic for some plant species and mammalian cell lines. Insensitivity of tomato to SAMs is determined by the *Alternaria* stem canker gene 1 (*Asc-1*), and sensitivity is associated with a mutated *Asc-1*. We show that SAM-sensitive species occur at a low frequency in the *Nicotiana* genus and that candidate *Asc-1* homologs are still present in those species. In *Nicotiana* spp., SAM-sensitivity and insensitivity also is mediated by a single codominant locus, suggesting that SAM-sensitive genotypes are host for *A. alternata* f. sp. *lycopersici*. *Nicotiana umbratica* plants homozygous for SAM-sensitivity are indeed susceptible to *A. alternata* f. sp. *lycopersici*. In contrast, SAM-sensitive genotypes of *Nicotiana spegazzinii*, *Nicotiana acuminata* var. *acuminata*, *Nicotiana bonariensis*, and *Nicotiana langsdorffii* are resistant to *A. alternata* f. sp. *lycopersici* infection concomitant with localized cell death. Additional (nonhost) resistance mechanisms to *A. alternata* f. sp. *lycopersici* that are not based on an insensitivity to SAMs are proposed to be present in *Nicotiana* species.

Plants have evolved mechanisms to recognize and respond to pathogens and to eliminate and negate the deleterious actions of fungal pathogenicity factors. As a result, compatible host–pathogen interactions are the exception (Johal et al. 1994). If no compatible interactions of the phytopathogen with genotypes of a plant (sub)species are known, resistance is

defined as broad, general, or nonhost resistance (Heath 1991). Is this type of resistance also effective against facultative (necrotrophic) fungal pathogens that produce phytotoxins? Necrotrophic fungal plant pathogens have the least sophisticated interaction with their hosts (Johal et al. 1994). Host cells are killed by the action of toxins. The dead host cells subsequently serve as nutrients for the development of the pathogen. The toxins are postulated to overrule nonhost resistance responses of plants by giving plant cells no opportunity nor time to effectively respond in advance of the spreading hyphae. If the toxins are toxic only to hosts susceptible to the fungus but not to resistant plants of the same species, they are defined as host-selective toxins (HSTs). In principle, the HSTs give similar disease symptoms when applied to healthy leaves of fungus-susceptible plant species (Yoder 1980). Most of the HSTs are small, secondary metabolites that are produced primarily by the fungal genera *Alternaria* and *Cochliobolus* (Walton 1996), but they also can be proteins (Ciuffetti et al. 1997). At least four lines of evidence have indicated that HSTs act as the primary pathogenicity determinants. First and most important, HST-deficient mutants are nonpathogenic (Ahn and Walton 1997; Akamatsu et al. 1997; Bukhalid et al. 1998; Ciuffetti et al. 1997; Macko et al. 1992; Navarre and Wolpert 1999; Tanaka et al. 1999; Walton 1996; Xiao et al. 1992; Yang et al. 1996). Second, plants that detoxify the HSTs are resistant to the pathogen (Multani et al. 1998). Third, expression of the HST-producing genes in toxin-deficient isolates or in related fungal species leads to pathogenicity on HST-sensitive hosts (Ciuffetti et al. 1997; Del Sorbo et al. 2000). Fourth, the addition of a HST to germinating nonpathogenic fungal spores leads to successful primary infections (Kodama et al. 1990; Xiao et al. 1992). Necrotrophic fungi also can make nonhost-selective toxins (non-HSTs) that are phytotoxic to a wider range of plants than the fungus infects (Walton 1996). Most HST-producing fungi have the basic aggressiveness to form appressoria or penetration pegs and, subsequently, penetrate healthy leaves of toxin-resistant nonhosts. Infections usually do not go beyond the first penetrated epidermal and mesophyll cells in nonhosts. Nonhost resistance to necrotrophic, HST-producing pathogens is presumed to be defined by the extreme insensitivity of nonhost plants to HSTs (Multani et al. 1998; Witsenboer et al. 1989).

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The *Alternaria alternata* f. sp. *lycopersici*-*Lycopersicon esculentum* (tomato) interaction provides a genetically and physiologically well-established system to study the role of HSTs in the negation of nonhost resistance (Brandwagt et al. 1998). Tomato genotypes that are sensitive to AAL toxins will support growth of the pathogen (Gilchrist et al. 1995). The secretion of AAL toxins is a genuine pathogenicity factor of *A. alternata* f. sp. *lycopersici* on tomato because toxin-deficient mutants cannot invade healthy tomato leaves (Akamatsu et al. 1997). AAL toxins and the *Fusarium moniliforme* fumonisins are sphinganine-analog mycotoxins (SAMs). They cause apoptosis in susceptible tomato lines and mammalian cell lines, presumably by inhibition of ceramide biosynthesis (Gilchrist et al. 1995; Wang et al. 1996a; Wang et al. 1996b). In tomato, insensitivity to SAMs is mediated by the *Alternaria* spp. stem canker (*Asc*) locus, which also mediates resistance to *A. alternata* f. sp. *lycopersici*. The tomato *Asc* locus has two alleles: *Asc* (insensitivity and resistance) and *asc* (susceptibility and sensitivity). The *Asc* locus inherits codominantly for toxin insensitivity and dominantly for fungus resistance (Van der Biezen et al. 1995). Recently, it was discovered that the single-copy gene *Asc-1* present at the *Asc* locus mediates SAM insensitivity in tomato. The *Asc-1* gene is homologous to the yeast longevity assurance gene LAG1 and has no homology to known plant resistance genes. Sensitivity to SAMs is associated with a dysfunctional *Asc-1* gene, and insensitivity presumably functions as a salvage pathway for ceramide-depleted plant cells (Brandwagt et al. 2000). Insensitivity or sensitivity to AAL toxins coincides with resistance or susceptibility, respectively, to *A. alternata* f. sp. *lycopersici* in the genus *Lycopersicon*, confirming the role of AAL toxins as host-specificity determinants (Van der Biezen et al. 1995). Apart from the genus *Lycopersicon*, sensitivity to AAL toxins occurs in the non-*Solanaceae* duckweed species *Lemna minor* and *Lemna paucicostata* (Abbas et al. 1994; Vesonder et al. 1992) and occurs at a low frequency in the *Solanoideae* subfamily of the *Solanaceae* spp. (Mesbah et al. 2000). It is not known, however, whether these toxin-sensitive species are susceptible to *A. alternata* f. sp. *lycopersici*. Sensitivity to AAL toxins clearly is the exception in plant species. Most species of the *Solanaceae*, *Arabidopsis thaliana*, and several other dicotyledonous or monocotyledonous species are resistant to 0.2 μ M AAL toxins, *A. alternata* f. sp. *lycopersici*, or both (Brandwagt et al. 1998; Mesbah et al. 2000).

Past studies on the host specificity of HST-producing fungi were performed by examination of a random and limited selection of plant species in a "nonhost" genus for sensitivity to the toxin or susceptibility to the pathogen. Our goal was to study nonhost resistance for a necrotrophic and HST-producing plant-pathogenic fungus on the plant-genus level to determine whether there were any AAL toxin and fumonisin B₁-sensitive species in a "nonhost" genus. Subsequently, the pathogenicity of AAL toxin-producing and -deficient *A. alternata* isolates on toxin-sensitive species could be assayed. The genus *Nicotiana* was chosen to study nonhost resistance to *A. alternata* f. sp. *lycopersici* for four reasons: i) *Nicotiana glutinosa*, *Nicotiana quadrivalvis*, and *Nicotiana tabacum* are resistant to the pathogen (Grogan et al. 1975); ii) the genus belongs to the *Cestroideae* subfamily, which originated in South America and is evolutionarily ancestral to and distant from the *Solanoideae* subfamily (Bogani et al. 1997; Olmstead and

Palmer 1992); iii) most *Nicotiana* species are available from seed repositories; iv) the evolutionary lineages have been thoroughly investigated in *Nicotiana* spp. (Bogani et al. 1997; Goodspeed 1954; Olmstead and Palmer 1992). Species of the *Suaveolentes* section of the *Petunioides* subgenus are unique to Australia and the Pacific region, most likely as a result of invasions from South America, thus providing species that do not occur in the native geographic distribution of *Lycopersicon* spp. (D'Arcy 1991). The genus contains approximately 72 species, after correcting for renamed species, which is acceptable for a complete inventory of the genus, similar to that of the *Lycopersicon* genus (Van der Biezen et al. 1995). In this paper, we show that SAM-sensitive species exist at a low frequency in the genus *Nicotiana*. SAM sensitivity and insensitivity are genetically and phenotypically similar, as they are in tomato. Surprisingly, four SAM-sensitive *Nicotiana* spp. genotypes tested resistant to *A. alternata* f. sp. *lycopersici*.

RESULTS

Previous studies of the host specificity of AAL toxins suggest that a low number of AAL toxin-sensitive *Nicotiana* spp. exist. Insensitivity and sensitivity to SAMs is displayed at all levels of plant development (Witsenboer et al. 1988), enabling a seed-germination bioassay to detect AAL toxin-sensitive species. In total, 122 accessions of 68 *Nicotiana* species were tested for their phenotype after germination on 0.1 μ M gibberellic acid-3 (GA₃) plus 0.2 μ M AAL toxins (Fig. 1). The assay covered 97% of the species in the genus *Nicotiana*, if subspecies were excluded. The addition of AAL toxins had no influence on the timing or efficiency of seedling germination. On the basis of the reaction of tested accessions to 0.2 μ M AAL toxins after the appearance of the root tip, the tested *Nicotiana* spp. were divided into three classes (Table 1). The largest class, class I (54 species), comprises 106 accessions that displayed similar root development and expansion of the (hypo)cotyl as compared with the water controls (Fig. 1A and B). This confirmed our expectation that most *Nicotiana* spp. are resistant to AAL toxins. In class II species, all tested accessions showed a clear inhibition of root elongation and root-hair formation at 0.2 μ M AAL toxins. Class II species, however, showed a normal chlorophyll development in the cotyledons. Class II is represented by seven species of three sections: *Nicotiana pauciflora* (Fig. 1C and D), *Nicotiana linearis*, *Nicotiana attenuata*, *Nicotiana nudicaulis*, *Nicotiana repanda*, *Nicotiana nesophila*, and *Nicotiana wigandoides* (Table 1). Detached leaves of the class II species showed no clear necrosis at 0.2 μ M AAL toxins (not shown). Class II species were therefore not analyzed further. In the most interesting class III species, at least one accession was found in which the root tip of all seedlings or a fraction of seedlings necrotized within 48 h (Table 2). The necrosis was similar to *asc,asc* tomato seeds in control germinations (data not shown). The seedlings with necrotic root tips were scored as sensitive (Fig. 1E and F). Plants of class III accessions subsequently were grown in the greenhouse to investigate whether the sensitivity of roots corresponded to the sensitivity of leaves. Detached leaves of class III toxin-sensitive species were tested in the leaflet bioassay that discriminates *Asc,asc* from *asc,asc* tomato genotypes. These leaves lost turgor after 48 h of incubation in 0.2 μ M AAL toxins, and the mesophyll

turned brown within an additional 24 h (Fig. 2), which is similar to the tomato control leaves. Although a limited number of individuals were tested, segregation ratios of the class III *Nicotiana* spp. accessions at 0.2 μ M AAL toxins were similar to those observed in the germination bioassay (Table 2). The leaves and seeds of AAL toxin-sensitive *Nicotiana* spp. genotypes also were sensitive to 2 μ M fumonisin B₁ (data not shown).

A single, codominant *Asc* locus is present in *Nicotiana* spp.

Crosses with the sensitive and insensitive class III *Nicotiana* spp. accessions were used to determine whether AAL toxin sensitivity and insensitivity in *Nicotiana* spp. are genetically similar as they are in *Lycopersicon* spp. Accessions of the species *Nicotiana acuminata* and *Nicotiana langsdorffii* were either completely sensitive or insensitive to AAL toxins in the germination bioassay. All individuals of the two *Nicotiana spegazzinii* accessions were sensitive to AAL toxins, thus genetic analysis with this species was impossible (Table 2). Sensitive and insensitive individuals were observed in single accessions of *Nicotiana umbratica* and *Nicotiana bonariensis*, suggesting that the plants used for the original seed

stocks were heterozygous for candidate *Asc-1* homologs (Table 2). Seeds from germ plasm repositories, however, usually are kept as pools (bulks) from a small population of plants to preserve genetic diversity of a self-pollinating species. The segregation ratios that were observed in the *N. umbratica* and *N. bonariensis* accessions (Table 2) did not necessarily reflect the zygosity or copy number of genes involved in SAM sensitivity and insensitivity.

Individual class III *Nicotiana* spp. plants (Table 2) that tested sensitive or insensitive in the leaf bioassay were first self-pollinated to determine the zygosity of candidate *Asc* loci. Subsequently, *N. umbratica* and *N. bonariensis* and *N. acuminata* and *N. langsdorffii* plants that tested homozygous for toxin insensitivity or sensitivity were reciprocally crossed to produce two F₁ populations (Table 2). The two F₁ populations subsequently were self-pollinated and test-crossed to homozygous, sensitive genotypes to determine the number of loci involved in toxin insensitivity. From Table 2 ($p < 0.1$) it can be concluded that AAL toxin insensitivity is determined by a single, dominant locus in *N. acuminata*, *N. bonariensis*, *N. umbratica*, and *N. langsdorffii*. The alleles of the *Nicotiana* spp. candidate *Asc* loci mediating toxin insensitivity and sensitivity will be referred to as *R* and *r*, respectively. If SAM

Fig. 1. AAL toxin germination bioassay of *Nicotiana* spp., which can be divided into three classes after **A, C, and E**, control germination on 0.1 μ M GA₃ and **B, D, and F**, germination on 0.1 μ M GA₃ + 0.2 μ M AAL toxins. Scale bars = 1 cm. **A and B**, In accessions of class I species, represented by *Nicotiana tabacum* cv. Petit Havana SR1, there were no obvious differences observed in the toxin treatment compared with the water controls. **C–D**, In accessions of class II species represented by *Nicotiana pauciflora* PI555546, inhibition of root growth and (hypo)cotyl elongation were observed in the toxin treatment, although the cotyledons emerged and became green. **E and F**, In accessions of class III species, represented by *N. umbratica* PI271993, a fraction of the roots became necrotic (arrows), whereas other seedlings had no obvious symptoms.

insensitivity also inherits codominantly in *Nicotiana* spp., the leaves of heterozygous (*Rr*) plants should have an intermediate phenotype, i.e., be sensitive to higher toxin concentrations. Detached leaves of *rr* and *Rr* *N. acuminata*, *N. bonariensis*, *N. spegazzinii*, *N. langsdorffii*, and *N. spegazzinii* genotypes responded similarly to *asc,asc* and *Asc,asc* *L. esculentum* detached leaves in serial dilutions of AAL toxins (data not shown). The responses of the leaves were variable, however, as a result of the differences in development and growth conditions among individual plants. Therefore, quantification of AAL toxin sensitivity in class III *Nicotiana* spp. *rr*, *Rr*, and *RR* genotypes was performed by measuring the 50% inhibitory concentration (IC_{50}) for root-growth inhibition by AAL toxins (Witsenboer 1991). It could be concluded that *rr*, *Rr*, and *RR* genotypes of the class III *Nicotiana* species have similar IC_{50} values as the tomato *asc,asc*, *Asc,asc*, and *Asc,Asc* control genotypes, except for *N. umbratica*, which tested more sensitive (Table 3). Thus, candidate *Asc* loci in class III *N. acuminata*, *N. langsdorffii*, and *N. umbratica* also inherit codominantly for toxin insensitivity.

***Nicotiana* species contain at least one candidate homolog of *Asc*.**

The phenotypes of the *r* and *R* alleles in class III *Nicotiana* spp. are similar to the tomato *asc* and *Asc* alleles, respectively, suggesting that the *R* alleles encode functional *Asc-1* homologs and the *r* alleles are dysfunctional. Class III *Nicotiana* spp. *rr* and *RR* genotypes were analyzed by low-stringency hybridization of DNA blots with the coding region of the *L. esculentum Asc-1* gene. The blot was washed at $2\times$ SSC at 60°C , which should allow for the identification of genes with 85% homology at the DNA level (Fig. 3). Under these conditions, previously undetected homologs of *Asc-1* became visible in both tomato *Asc* isogenic lines. After a higher stringency wash ($0.5\times$ SSC, 60°C , 92% homology), only one fragment (two for the *RR N. langsdorffii*) still hybridized in all *Nicotiana* spp. genotypes tested. In *N. acuminata* and *N. um-*

bratica, no obvious restriction fragment length polymorphisms (RFLPs) were detected among the *rr* and *RR* genotypes. In *N. langsdorffii*, however, rearrangements in candidate *Asc-1* loci were detected. The *rr* genotype lacks one copy of a candidate *Asc-1* homolog, and the remaining fragments showed RFLPs. *N. bonariensis* contains the least homologous sequences. An autoradiogram after the $0.5\times$ SSC wash showed one similar, weakly hybridizing fragment for the *Rr* and *rr* genotypes. From the DNA blots it could be concluded that candidate *Asc-1* homologs occur in class III *Nicotiana* spp. and that *N. langsdorffii* has obvious rearrangements in a candidate *Asc* locus.

Four AAL toxin-sensitive *Nicotiana* spp. are nonhost for *A. alternata* f. sp. *lycopersici*.

To test whether sensitivity to AAL toxins coincides with susceptibility to the pathogen, class III *Nicotiana* spp. genotypes (Table 2) were inoculated with *A. alternata* f. sp. *ly-*

Fig. 2. Detached leaf bioassay of the *Nicotiana* spp. homozygous insensitive (*RR*) and homozygous sensitive (*rr*) genotypes. Sensitivity to AAL toxins was assessed by incubation of detached *Nicotiana* spp. leaves on filter papers, with or without $0.2\ \mu\text{M}$ AAL toxins. Reaction of the leaves of class III *Nicotiana umbratica rr* and *RR* genotypes are shown. The *rr* genotype reacted with a typical necrosis, also observed for *asc,asc* detached tomato leaflets (data not shown), whereas the *RR* genotype showed no obvious symptoms upon incubation in AAL toxins.

Table 1. AAL toxin germination bioassay of the genus *Nicotiana*^a

Subgenus	Section	Tested	Class I	Class II	Class III	Not Tested ^{b,c}
<i>Petunioides</i>	<i>Acuminatae</i>	7	2	3	2	1 ^b
	<i>Alatae</i>	9	7		2	
	<i>Bigelovianae</i>	3	3			
	<i>Noctiflorae</i>	3	3			1 ^b
	<i>Nudicaules</i>	1		1		
	<i>Repandae</i>	3	2	1		
	<i>Suaveolentes</i>	21	20		1	1 ^c
	<i>Trigonophyllae</i>	2	2			
	<i>Undulatae</i>	3	2	1		
<i>Rustica</i>	<i>Paniculatae</i>	7	7			
	<i>Rusticae</i>	1	1			
	<i>Thyrsoflorae</i>	0				1 ^c
<i>Tabacum</i>	<i>Genuinae</i>	1	1			
	<i>Tomentosae</i>	7	7			
Total		68	57	6	5	4

^a The *Nicotiana* spp. were classified on basis of their reaction to $0.2\ \mu\text{M}$ AAL toxins after the appearance of the root tip. Class I species displayed no inhibition of root growth, normal root hair development, and expansion of the (hypo)cotyl. Class II species showed inhibition of root elongation and root hair formation but had normal chlorophyll development in the hypocotyls. Class III species contain at least one accession with sensitive seedlings. In sensitive seedlings, the root tip necrotized within 48 h and root growth ceased similar to *asc,asc* tomato seeds in control germinations. The total number of species tested from one *Nicotiana* spp. section and the division of the total number in the above classes are represented.

^b Seeds of *Nicotiana ameghinoi* and *Nicotiana longibracteata* were not available.

^c Seeds of *Nicotiana fragrans* and *Nicotiana thyrsoflora* did not germinate.

copersici under optimal conditions for disease development. The saprophytic *A. alternata* isolate did not induce visual symptoms in three independent experiments on any of the SAM-sensitive tomato or *Nicotiana* spp. plants. The pathogenic *A. alternata* f. sp. *lycopersici* infected all upper parts of *asc,asc* tomato plants, as described (Grogan et al. 1975), provided that relative humidity was close to 100% to ensure spore germination and penetration in the first 3 days. No visual symptoms were observed on control *Asc,Asc* and *Asc,asc* tomato plants. *N. umbratica* *rr* genotypes were susceptible to *A. alternata* f. sp. *lycopersici*, although the symptoms were more severe than on tomato *asc,asc* plants. Enlarging water-soaked, wilted lesions appeared in *rr* *N. umbratica* plants 1 week postinoculation, and the spread of fungus on the upper

parts of the plants was observed. The plants completely wilted 7 to 14 days postinoculation, and the fungus sporulated in water-soaked areas (Fig. 4C and D). In contrast, tomato *asc,asc* control plants died as a result of typical stem cankers. Water-soaked lesions did not occur on *Rr* *N. umbratica* plants (Fig. 4C and F), although occasional necrotic areas were observed on young parts that accumulated condensation and spores after the inoculation. Surprisingly, local lesions appeared in *rr* genotypes of *N. bonariensis*, *N. spegazzinii*, and *N. acuminata* var. *acuminata* within 7 days after infection with *A. alternata* f. sp. *lycopersici* (Fig. 4A). Superficial growth of the fungus and spreading necrosis, as observed in *asc,asc* tomato plants, did not occur. Moreover, sporulation of the fungus from the necrotic spots, as observed in *rr* tomato leaflet, was not present in these *Nicotiana* spp. After visualization of the fungus with lactophenol blue, it was clear that the fungus did not grow beyond the lesions (not shown). The stem tissues and noninoculated *Nicotiana* spp. leaves (Fig. 4B) showed no apparent signs of toxin action or (secondary) fungal infestation as observed in tomato control plants (Fig. 4E). *N. langsdorffii* *rr* plants had an intermediate phenotype. The infection spread on leaf surfaces completely covered with run-off condensation water. The infection stopped, large sunken lesions were formed, and growth of the fungus ceased, however, when humidity dropped after removal of the plastic cover. *N. acuminata* and *N. langsdorffii* *Rr* genotypes had an intermediate number of lesions, and *N. bonariensis* *Rr* genotypes had no visible lesions. In all three spore inoculation assays performed, *Rr* and *RR* *Nicotiana* spp. plants were resistant to *A. alternata* f. sp. *lycopersici* (Table 3).

DISCUSSION

A single-Mendelian locus for AAL toxin-insensitivity inherits in a codominant fashion in the genus *Nicotiana*. Thus, the genus *Nicotiana* phenotypically and genotypically behaves like the genus *Lycopersicon* with respect to SAM insensitivity. Heterozygous genotypes (*Rr*) of *N. acuminata* and *N. langsdorffii* show necrotic lesions upon spore inoculation, despite similar AAL toxin sensitivity of the heterozygous leaves compared with tomato. Until now, most genera, except for the genus *Lycopersicon*, were believed to be nonhost for *A. alternata* f. sp. *lycopersici* on the basis of their insensitivity to AAL toxins. This paper provides support for screens on the plant-genus level (Kamoun et al. 1993; Kamoun et al. 1998; Laugé et al. 1998) to study nonhost resistance to necrotrophic pathogens. With a screen on the genus level it became clear that certain *Nicotiana* spp. are host for *A. alternata* f. sp. *lycopersici*, as demonstrated by the sensitivity of the *rr* genotype of *N. umbratica*. Sensitivity of *Nicotiana* spp. to AAL toxins, however, does not automatically lead to susceptibility to *A. alternata* f. sp. *lycopersici*, as is the case in the *Lycopersicon* genus. Based on these observations, several questions can be asked. First, can AAL toxins still be considered as HSTs? Second, how would SAM sensitivity be maintained in plant species from an evolutionary perspective? For instance, has there been selection on the presence of SAM insensitivity? Third, what would be the mechanism behind the resistance to *A. alternata* f. sp. *lycopersici* in SAM-sensitive *Nicotiana* spp.?

Previous studies indicated that susceptibility to AAL toxins is not restricted to the genus *Lycopersicon* and that additional

Fig. 3. Low-stringency DNA blot to detect candidate *Asc* homologs in class III *Nicotiana* spp. Lane 1, *Lycopersicon esculentum* homozygous sensitive *asc,asc* (*r*) and resistant *Asc,Asc* (*R*) isogenic lines. Lanes 2–6, Homozygous sensitive *rr* (*r*) and insensitive *RR* (*R*) *Nicotiana* spp. genotypes, except for *Nicotiana bonariensis*, where a heterozygous *Rr* (*H*) genotype was used. Lane 2, *Nicotiana acuminata* var. *acuminata* NIJ 974750095 (*r*) and *N. acuminata* PI42347 (*R*). Lane 3, *N. bonariensis* PI555489 (*r*) and (*H*). Lane 4, *Nicotiana langsdorffii* PI42337 (*r*) and PI555529 (*R*). Lane 5, *Nicotiana umbratica* NIJ974750102 (*r*) and (*R*). Lane 6, *Nicotiana spegazzinii* NSL75788 (*r*). In tomato, the region adjacent to the promoter of the single-copy *asc-1* mutant allele contains several deletions, although they have no effect on the transcription level relative to *Asc-1* (Brandwagt et al. 2000). *Hind*III digestion was used to detect a 300-bp deletion in the upstream region of *asc,asc* tomato plants (lane 1r). Candidate *L. esculentum* *Asc-1* homolog(s) larger than 5.0 kb are indicated with horizontal arrows; only the largest fragment remained visible after a 0.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) wash. Hybridizing *Nicotiana* spp. fragments still visible after a 0.5× SSC wash at 60°C are indicated with small arrows.

Solanaceous and non-*Solanaceous* spp. also are sensitive (Abbas et al. 1994; Mesbah et al. 2000). It has not been tested, however, whether these sensitive species are susceptible to fungal infection. Four AAL toxin-sensitive *Nicotiana* spp. are resistant to the pathogen. Thus, the AAL toxins may not be classical HSTs on the basis of earlier definitions (Walton 1996; Yoder 1980). Could AAL toxins be considered non-

HSTs? The currently known non-HSTs are toxic to a wide range of plants. SAMs, however, are toxic only to a limited number of plant species. In addition, the (non)HST-producing *Alternaria longipes*, which causes brown spot of tobacco (Stavelly et al. 1971), does not produce AAL toxins in vitro. As a result, *A. longipes* is nonpathogenic on *asc. asc* tomato cultivars (Gilchrist et al. 1992; Grogan et al. 1975). Based on the

Fig. 4. Infection of greenhouse-grown tomato and *Nicotiana* spp. plants with *Alternaria alternata* f. sp. *lycopersici*. **A, C, and E,** Typical symptoms of detached leaves or **B, D, and F,** whole plants were photographed ten days postinoculation. Scale bars = 1cm. Genotypes of the (candidate) *Asc* loci present in the inoculated plants are indicated with *R* (*Asc*, toxin resistance) and *r* (*asc*, toxin sensitivity). **A and B,** Of the four AAL toxin-sensitive, fungus-resistant *Nicotiana* spp., *Nicotiana acuminata* var. *acuminata* is shown. **C and D,** *N. umbratica* *rr* genotype is sensitive to 0.2 μM AAL toxins and susceptible to *A. alternata* f. sp. *lycopersici*. **C and F,** *N. umbratica* *RR* and *Rr* genotypes are not sensitive to 0.2 μM AAL toxins and *A. alternata* f. sp. *lycopersici*. **E,** *Lycopersicon esculentum* *Asc* isolines controls were either completely resistant or sensitive to *A. alternata* f. sp. *lycopersici*. Only detached leaflets of the *RR* (left three leaflets) and *rr* (right three leaflets) genotypes are shown.

lack of pathogenicity of AAL toxin-deficient mutants (Akamatsu et al. 1997) and the fact that only the SAM-sensitive *L. esculentum*, *Lycopersicon cheesmanii*, and *N. umbratica* can be hosts for the fungus, the AAL toxins should still be regarded as HSTs. The action of AAL toxins, however, could be overruled by other mechanisms, as discussed below.

Candidate homologs of *Asc* and *asc* (*R*- and *r*-) alleles coexist in nature among and within *Solanaceous* spp. outside of tomato (Tables 1 and 2), the host from which the fungus was isolated originally (Grogan et al. 1975). The class III *Nicotiana* spp. have different geographic origins in South America and Australia and do not form a clear lineage, according to the classical evolution diagram of Goodspeed (1954). Thus, SAM sensitivity appears to be distributed randomly in the genus *Nicotiana*. Moreover, the presence of *r* and *R* alleles in *Nicotiana* spp. plants does not lead to obvious pleiotropic effects in standard greenhouse conditions, and four SAM-sensitive *Nicotiana* spp. are resistant to the fungus. The *Asc* and *asc* alleles came from tomato-breeding programs in areas where the disease was not present. Moreover, *asc* predated the disease, and *Asc* was incorporated in the absence of the disease (Gilchrist et al. 1995). On the basis of the aggressiveness of the fungus on *N. umbratica*, however, selection pressure on the *r* allele in *N. umbratica* populations is conceivable. Until now, *A. alternata* f. sp. *lycopersici* has been found only in California and the Mediterranean countries where tomatoes are grown in the field. If *A. alternata* f. sp. *lycopersici* would occur in northwest Australia, would it be possible that the *r* and *R* alleles coexisted? The arms-race model in which variation in disease resistance is transient and host populations are

monomorphic at *R*-gene loci has been rejected as a result of studying the *Rpm1* gene in a large number of *A. thaliana* ecotypes (Stahl et al. 1999). The susceptible and resistant alleles of the *A. thaliana Rpm1* gene have coexisted for millions of years, which is characteristic of the considerable polymorphisms found at other *R*-gene loci in plant populations. Thus, even in the presence of the pathogen, susceptible allele(s) can be stably inherited in a population of plants, explaining the existence of *rr N. umbratica* genotypes, even in the possible presence of *A. alternata* f. sp. *lycopersici*. Alternatively, the *R* alleles could not be fixed completely in *N. umbratica*. Without the selection pressure of the fungus, the next reason for the existence of *rr* plants could be their enhanced fitness under natural conditions, as compared with *RR* plants.

In *L. esculentum*, sensitivity is associated with a deletion in the second exon of the *asc-1* gene leading to a predicted dysfunctional ASC protein (Brandwagt et al. 2000). The loss-of-function mutation of the *asc-1* allele leads to no pleiotrophic effects in tomato. *L. pennellii* contains a single dominant *Asc* locus, which maps to the same position as the *L. esculentum* *Asc* locus and mediates a high level of resistance to SAMs and *A. alternata* f. sp. *lycopersici* (Van der Biezen et al. 1995). Candidate *Asc* homologs are present in class III *Nicotiana* spp. Whether these candidates are allelic may be determined by interspecific crosses (Goodspeed 1954). Functional SAM insensitivity is observed in most *Solanaceous* spp. Together, these above phenomena closely resemble the widespread resistance to *Cochliobolus carbonum* race 1 and its host-selective HC-toxin in monocotyledonous plants species possessing candidate *Hm* homologs and HC-toxin reductase activity (Multani et al. 1998). By analogy, the DNA sequence

Table 2. Genetic analysis of AAL toxin-insensitivity and sensitivity in class III *Nicotiana* species^a

Species	2n =	Accession	Bioassay ^b		Selfed progeny ^c	Generation <i>Rr</i> plants	<i>Rr</i> plants	
			Germination	Detached leaf			Selfed progeny	Test cross (x <i>rr</i>)
<i>N. acuminata</i>	24	PI555471	47:0	12:0	57:0	x NIJ964750095	76:28	29:24
		PI42347	52:0	12:0	87:0	x NIJ964750095	84:29	42:41
<i>N. acuminata</i> cv. <i>acuminata</i>	24	NIJ964750095	0:34	0:13	0:50	x PI555471	55:17	41:45
<i>N. bonariensis</i>	18	PI555489	5:5	16:9	None	^d	54:18 ^e	15:18
<i>N. langsdorffii</i>	18	PI42337	0:63	0:8	0:69	x PI555529	64:18 ^e	48:49
		PI555529	54:0	8:0	56:0 ^e	x PI42337	60:19 ^e	67:65
<i>N. spegazzinii</i>	24	NSL 75788	0:60	0:5	0:50			
		PI281756	0:48	0:10	0:41			
<i>N. umbratica</i>	46	NIJ974750102	30:9	13:14	<i>Rr</i> 98:23 <i>rr</i> 0:95 <i>RR</i> 85:0 ^f	x <i>RR</i> x <i>rr</i>	100:32 112:40	54:49 56:52
		NSL 75792	12:12	9:15				
		PI271993	167:36	22:3	^f			

^a Segregation ratios of seeds and plants were scored in bioassays using 0.2 μ M AAL toxins are depicted as insensitive–sensitive and never deviated significantly from expected assuming $p < 0.1$ if $\chi^2 > 2.7$. All species are diploid (diploid chromosome number is in column 2). For the selfed progeny and test crosses, results from a typical representative of three plants are shown.

^b Bioassays were performed on seeds received from the germ plasm repositories.

^c Segregation ratios were determined with a germination bioassay.

^d Heterozygous plants were identified after test cross with a sensitive genotype, resulting in a 1:1 segregation of the progeny.

^e Heterozygous individuals were crossed to circumvent female gametophytic incompatibility.

^f Segregation ratios of selfed plants were similar to those of NIJ974750102 ($n > 60$), a progeny of the PI271993 seed stock. Therefore, these accessions were not analyzed further.

and expression of the *Asc-1* homologs of *RR* and *rr* genotypes of class III *Nicotiana* spp. could reveal the molecular basis of sensitivity and provide clues about events in evolution that lead to sensitivity. Complicating factors will be the low expression level of *Asc-1* and the low sequence conservation among the known *Asc-1* homologs (Brandwagt et al. 2000). These factors will require heterologous hybridization screens of genomic *Nicotiana* spp. libraries to identify candidate *Nicotiana* spp. *Asc-1* homologs. With the use of segregating populations of *RR* and *rr* *N. langsdorffii* genotypes, the RFLPs identified (Fig. 3) may facilitate the molecular isolation of the candidate *Asc* homolog(s) involved in SAM insensitivity.

Apart from the evolutionary aspects of the *Nicotiana* spp.–*A. alternata* f. sp. *lycopersici* interactions, it is still enigmatic how SAM-sensitive *Nicotiana* spp. can resist *A. alternata* f. sp. *lycopersici* infection. To produce a sufficient amount of HSTs, the fungus has to grow on nutrients released from the dead cells in the primary lesions. If the fungus is inhibited on these primary lesions, only the toxins from the germinating conidia in the spore drops will develop necrotic spots. The limited ramification of the fungus can be explained by two hypotheses. First, in resistant *Nicotiana* spp. leaves, antifungal compounds could be released and induced during cell death invoked by AAL toxins. Second, our observations might be explained by a rapid recognition of a nonspecific *Alternaria* spp. elicitor of HR that results in the death of the plant cells before the transport of the AAL toxins and further growth of the pathogen can occur. Although it is still controversial if HR functions in disease resistance to fungal pathogens (Richael and Gilchrist 1999), there are examples when HR can function to limit systemic growth of fungal phytopathogens. The expression of the *Phytophthora cryptogea* elicitor cryptogein under a pathogen-inducible promotor in tobacco leads to localized necrosis and enhanced resistance to the unrelated pathogens *Erisiphe cichoracearum*, *Thielaviopsis basicola*, and *Botrytis cinerea* (Keller et al. 1999). It remains questionable whether the HR-like response observed in our experiment contributes to the limitation of *A. alternata* f. sp. *lycopersici* growth or that these lesions are caused by our inoculation procedures. The accumulation of UV fluorescence immediately adjacent to the necrotic spots, similar to that described by Dorey et al. (1997), and cell-wall appositions in the cells of the lesion (not shown) suggest a genuine HR. In *N. tabacum*,

however, recognition of *Tobacco mosaic virus* and *Pseudomonas syringae* pv. *phaseolicola* leads to a HR that bears the characteristics of apoptosis (Mittler et al. 1997) similar to AAL toxin-induced cell death of tomato (Wang et al. 1996b). Cell death as a result of HR or cell death by the action of AAL toxins thus cannot be discriminated in our model system. Consequently, the standard procedures to detect HR-like cell death or the expression level of HR-induced genes were not used. Our data suggest that nonhost resistance responses may overrule the action of HSTs. In conclusion, we propose that additional mechanisms besides insensitivity to HSTs can determine whether a plant is host for an HST-producing phytopathogen. It will be challenging to elucidate the molecular basis of these nonhost resistance mechanisms.

MATERIALS AND METHODS

Plant and fungal materials.

Paired tomato lines isogenic and homozygous for the *Asc* locus (the *Asc* [resistant] and *asc* [sensitive] isolines) (Clouse and Gilchrist 1987) were used as controls for bioassays and DNA analysis. Seeds of *Nicotiana* spp. were donated by the U. S. National Plant Germplasm System at the National Seed Storage Laboratory, Ft. Collins, CO, U.S.A.; the Tobacco Collection at the Crop Science Department, Oxford Tobacco Research Station, North Carolina State University, Oxford, U.S.A.; and the Botanical Garden of the University of Nijmegen, The Netherlands. *Nicotiana simulans* was later determined to be *N. acuminata* var. *acuminata* on its leaf and flower morphology, explaining why a subspecies has been included. Detailed information on the *Nicotiana* spp. accessions can be obtained from online databases at the U. S. Department of Agriculture, Agricultural Research Service, Germplasm Resources Information Network (Beltsville, MA, U.S.A.), and at the Botanical Garden of Nijmegen. Isolates of the pathogenic *A. alternata* f. sp. *lycopersici* AS27-3 (Clouse and Gilchrist 1987) and a nonpathogenic *A. alternata* (Gilchrist et al. 1992) were maintained routinely on potato dextrose agar and checked for (non-)pathogenicity on *asc,asc* control tomato plants (Gilchrist and Grogan 1975). The pathogenic *A. alternata* f. sp. *lycopersici* is likely related to *A. alternata* (Gilchrist et al. 1992), although *A. alternata* f. sp. *lycopersici* has been proposed to be renamed to *Alternaria arborescens* (Simmons 1999).

Table 3. Responses of *Lycopersicon esculentum* and class III *Nicotiana* species to AAL toxins and *Alternaria alternata* f. sp. *lycopersici* infection^a

Species	IC ₅₀ AAL toxins (μm)			<i>A. alternata</i> f. sp. <i>lycopersici</i> infection		
	<i>rr</i>	<i>Rr</i>	<i>RR</i>	<i>rr</i>	<i>Rr</i>	<i>RR</i>
<i>L. esculentum</i>	0.12	0.3	3.8	Stem cankers, interveinal necrosis	–	–
<i>N. acuminata</i>	0.07	1.6	3.2	HR	HR ^b	–
<i>N. bonariensis</i>	Not determined	Not determined	Not determined	HR	–	Not determined
<i>N. langsdorffii</i>	0.01	0.17	3.5	HR ^c	HR ^b	–
<i>N. spegazzinii</i>	0.04	Not available	Not available	HR	Not available	Not available
<i>N. umbratica</i>	0.0054	0.032	0.77	Wilting, expanding necrosis	–	–

^a For the 50% inhibitory concentration (IC₅₀) determination, seeds were germinated on different concentrations of AAL toxins, and the half-maximum concentration required for complete root growth inhibition was determined essentially as described by Witsenboer et al. 1991. The symptoms after *A. alternata* f. sp. *lycopersici* infection were determined 10 days postinoculation of greenhouse-grown plants incubated in conditions optimal for disease development. HR = hypersensitive-like necrotic spots. – = no obvious symptoms

^b Hypersensitive-like spots, less abundant than on *rr* genotype.

^c Necrotic lesions expand at 100% humidity but stop at 60% humidity.

In vitro bioassays.

Cell-free culture filtrate (CFCF) of *A. alternata* f. sp. *lycopersici* was produced as described (Gilchrist and Grogan 1975). A 95–5% mixture of AAL toxins T_A–T_B was purified from the CFCF (Clouse et al. 1985). Concentrations of AAL toxins were determined relative to glycine standards in 96 well plates by O-phthalaldehyde derivatization (Witsenboer 1991) and subsequent measurement in a fluorometer. Approximately 40 seeds were germinated in 9-cm petri dishes on no. 595 S&S filter papers (Schleicher & Schuell, Dassel, Germany) soaked in 4 ml of 0.1 μ M gibberellic acid-3 (GA₃) supplemented with 0.2 μ M purified AAL toxins. All tested *Nicotiana* spp. accessions had a synchronous control germination in 0.1 μ M GA₃, except for *Nicotiana fragrans* and *Nicotiana thyrsoflora*, which were recalcitrant to germination. The plates were sealed because differences between sensitivity and insensitivity were less pronounced if the plates dried accidentally during germination. The addition of GA₃ to synchronize germination did not potentiate or inhibit AAL toxin-induced cell death because class III accessions sown on AAL toxins without GA₃ had similar phenotypes and segregation ratios for AAL toxin sensitivity. Symptoms were scored 10 days after sowing when the elongating roots of toxin-resistant class I seedlings had root hairs and the root tips of sensitive seedlings showed a brown necrosis.

Standard leaflet bioassays were performed in triplicate according to Gilchrist et al. (1992) by incubation of detached leaflets of 6-week-old plants in 9-cm petri dishes on S&S filter papers soaked with 3 ml of 0.2 μ M AAL toxins mixture. Leaf necrosis development, starting between the veins and eventually spreading through the whole leaf, was scored 72 h after incubation in a climate cabin (22°C) in the light (3 kilolux) to minimize variation. To test the sensitivity of *Nicotiana* spp. relative to the tomato *Asc* isogenic lines, leaves were incubated in a series of 1,000, 300, 100, 30, 10, 3, and 0 nM-purified AAL toxins, and the leaf-sensitivity index was determined as described (Van der Biezen et al. 1995). The IC₅₀ for inhibition of root growth by AAL toxins was estimated by measuring root lengths 7 days after sowing on the above concentration series of purified AAL toxins supplemented with 0.1 μ M GA₃, essentially as described (Witsenboer 1991).

Fungal infection assay.

The *Alternaria* spp. isolates were grown for 14 days on V8 plates (20% vol/vol V8 juice [Campbell Soup, Camden, NJ, U.S.A.], 30 mM CaCO₃, and 1.6% agar) at 25°C under continuous near-UV light for profuse sporulation. Spore suspensions were made by releasing the spores with 0.05% vol/vol Tween-80 in water with a Dragalski spatula. Spores were filtered to remove mycelial debris, centrifuged, washed twice in tap water to remove in vitro-produced toxins, and diluted to a final concentration of 10⁶ spores per ml (Grogan et al. 1975). *Nicotiana* spp. and tomato seeds were pregerminated in petri dishes and transferred to 1-liter pots containing a 50–50 vol/vol mixture of quartz sand and potting soil and grown under standard Dutch greenhouse conditions (20°C, 60% humidity, 15 kilolux light intensity maintained by automated artificial lights) for 4 weeks. Plants were covered by a plastic tunnel and pre-incubated until humidity reached 100%. Inoculation was performed with 10 ml of inoculum per plant with a household plant sprayer. Plants were incubated for 3

days at 20°C in temperate light (1 kilolux) and maximum humidity. After removal of the plastic tunnel, conditions became standard again. Disease symptoms were scored 10 days after inoculation when *asc,asc* control tomato plants had developed leaf necrosis and stem cankers. To rule out seasonal effects and variations among experiments, inoculations were performed three times (February, June, and October).

Molecular genetic analysis.

Controlled crosses were performed with greenhouse-grown plants by standard emasculating and pollination techniques. In general, *N. bonariensis* crosses resulted in a low seed content, and only a minor fraction of the crosses produced viable seeds, making genetic analysis of this species cumbersome. Accessions *N. bonariensis* PI555489 and *N. langsdorffii* PI555529 could not be self-pollinated as a result of female gametophytic sterility. In later crosses, the sterility inherited dominantly, which is typical for the *Solanaceae* spp. To circumvent the self-incompatibility of *N. langsdorffii* and *N. bonariensis*, plants that scored sensitive or putatively heterozygous in the leaflet bioassay were crossed to obtain artificial selfed progeny. Segregation ratios of selfed progeny and crosses were determined by the seed germination bioassay. For DNA analysis, tomato and *Nicotiana* spp. plants were grown in the greenhouse, and mature leaves were harvested 6 weeks after emergence of the seedlings. A DNA blot with *Eco*RI- or *Hind*III-digested genomic DNA (10 μ g) of these leaves was prepared with positively charged nylon membranes (Hybond N⁺, Amersham International, Little Chalfont, Buckinghamshire, U.K.) as described (Van der Biezen et al. 1996). The DNA blot was hybridized at 55°C with a radiolabeled, 1.5-kb polymerase chain reaction fragment encompassing the complete *Asc-1* open reading frame prepared by amplification with the oligonucleotides BASC86 (forward) CGGG ATCCCGATCAGTCTTTGTGGTCATCATC and BASC87 (reverse) GGAATTCCTGCAATTCATTTGAACTACAAC (Brandwagt et al. 2000), washed at increasing stringency at 60°C (5, 2, and 0.5 $\times$ SSC [1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate]), and after each wash analyzed with a PhosphorImager system (Molecular Dynamics).

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