Overexpression of the Tomato *Asc-1* Gene Mediates High Insensitivity to AAL Toxins and Fumonisin B₁ in Tomato Hairy Roots and Confers Resistance to *Alternaria alternata* f. sp. *lycopersici* in *Nicotiana umbratica* Plants

Bas F. Brandwagt, Tarcies J. A. Kneppers, H. John J. Nijkamp, and Jacques Hille

Department of Genetics, Free University, Institute for Molecular Biological Sciences, BioCentrum Amsterdam, De Boelelaan 1087, 1081 HV, Amsterdam, The Netherlands

Submitted 9 May 2001. Accepted 5 September 2001.

The sphinganine-analog mycotoxins (SAMs) fumonisin B₁ and AAL toxins are inhibitors of eukaryotic sphinganine *N*-acyltransferase in vitro. Treatment of eukaryotes with SAMs generally results in an accumulation of sphingoid base precursors and a depletion of complex sphingolipids. The *asc,asc* genotypes of tomato (*Lycopersicon esculentum*) and *Nicotiana umbratica* are sensitive to SAMs and host of the AAL toxin-producing fungus *Alternaria alternata* f. sp. *lycopersici*. Codominant insensitivity to SAMs in tomato is mediated by the *Asc-1* gene, and sensitivity is associated with a frame-shift mutation present in *asc-1*. We investigated the function of *Asc-1* in mediating insensitivity to SAMs and resistance to the fungus by overexpression of *asc-1* and *Asc-1*. In this study, it is shown that overexpression of these genes did not lead to visual symptoms in tomato hairy roots and *N. umbratica* plants. Overexpression of *asc-1* did not influence the (in)sensitivity to SAMs. Overexpression of *Asc-1* in SAM-sensitive hairy roots and *N. umbratica* plants, however, mediated a high insensitivity to SAMs and resistance to plant infection by *Alternaria alternata* f. sp. *lycopersici*.

Additional keywords: apoptosis, ceramide, *Fusarium moniliforme*, LAG1, stem canker.

Mycotoxins can be virulence factors of plant-pathogenic fungi or postharvest contaminants of agricultural products. Therefore, these toxins create a health hazard for humans and livestock (Desjardins and Hohn 1997; Walton 1996). One class of mycotoxins structurally resembles sphinganine, an intermediate of sphingolipid biosynthesis (Wang et al. 1996a). This class is defined as sphinganine-analog mycotoxins (SAMs) and

includes the AAL toxins of the tomato pathogen *Alternaria alternata* f. sp. *lycopersici* (Wang et al. 1996b) and the fumonisins of *Fusarium* species present on cereals (Norred et al. 1999). AAL toxin is a true pathogenicity factor of *A. alternata* f. sp. *lycopersici*, because toxin-deficient natural variants or laboratory mutants cannot grow on living tomato plants (Akamatsu et al. 1997). Fumonisin production in *Fusarium moniliforme*, however, is not required to infect maize ears (Desjardins and Plattner 2000). The fumonisin B₁ (FB₁) and AAL toxin T_A isomers have been most intensively studied in plant and nonplant model systems (Brandwagt et al. 1998; Merrill et al. 1997; Stone et al. 2000; Wang et al. 1996a; Wu et al. 1995). Sphinganine *N*-acyltransferase (SAT) is competitively inhibited by nanomolar concentrations of FB₁ in enzyme preparations of every eukaryotic cell type tested, including plants (Lynch 2000; Merrill et al. 1996). In plant leaves, roots, and protoplasts, the inhibition of SAT leads predominantly to growth inhibition and, in SAM-sensitive genotypes, to apoptosislike cell death (Brandwagt et al. 1998). A small number of *Solanaceous* species in the genera *Lycopersicon*, *Solanum*, and *Nicotiana* contain SAM-sensitive genotypes (<1 µM), whereas monocotyledons generally resist concentrations above 100 µM of AAL toxins (Abbas et al. 1995; Brandwagt et al. 2001; Mesbah et al. 2000; van der Biezen et al. 1995). Sensitivity to SAMs also occurs in the non-*Solanaceous* duckweed species *Lemna minor* and *Lemna paucicostata* (Abbas et al. 1994; Vesonder et al. 1992) but is clearly the exception in plant species.

Our interest is the study of SAMs as pathogenicity factors of necrotrophic fungal plant pathogens. Insensitivity to SAMs by tomato and by several *Nicotiana* species is mediated by the single codominant *Alternaria* stem canker (*Asc*) locus (Brandwagt et al. 2001). The molecular isolation of the tomato *Asc-1* gene mediating insensitivity to AAL toxins and FB₁ in hairy roots (Brandwagt et al. 2000) was a step forward in understanding the toxic principles of SAMs in plants. The *Asc-1* gene is homologous to the yeast longevity assurance gene (*LAG1*), the expression of which decreases with replicative age and increases life span by 50% compared with an $\Delta lag1$ strain (D'Mello et al. 1994). In concert with its homolog LAC1, the endoplasmic reticulum (ER)-localized LAG1 protein facilitates ER to Golgi transport of glycosylphosphatidylinositol (GPI)-anchored proteins, which is also sphingolipid dependent (Barz

Corresponding author: Jacques Hille; E-mail: j.hille@biol.rug.nl

Current address of B. F. Brandwagt: Laboratory of Phytopathology, Wageningen University and Research Center, Marijkeweg 22, 6709 PG, Wageningen, The Netherlands.

Current address of J. Hille: Department Molecular Biology of Plants, University of Groningen, Kerklaan 30, 9751 NN, Haren, The Netherlands.

*The e-Xtra logo stands for "electronic extra" and indicates the HTML abstract available on-line contains supplemental material not included in the print edition.

and Walter 1999; Muniz et al. 2001; Skrzypek et al. 1997). The human and *Caenorhabditis elegans* LAG1 homologs can functionally complement the lethal phenotype of a *Lag1/Lac1* double deletion in yeast (Jiang et al. 1998) and the LAG1 family is widespread in eukaryotes (Brandwagt et al. 2000), suggesting a universal function for LAG1-like proteins. The *Asc-1* gene has at least two distant homologs in tomato (Brandwagt et al. 2001) and three possible homologs in the *Arabidopsis thaliana* genome (The Arabidopsis Genome Initiative 2000). Although functional redundancy can be expected by analogy with yeast, sensitivity to SAMs in leaves and roots of the *asc,asc* isogenic tomato line is associated with a single frame-shift mutation in the

Asc-1 gene (= *asc-1*), and the *Asc-1* gene alone is sufficient to complement the SAM sensitivity (Brandwagt et al. 2000). It is still enigmatic how the *Asc-1* gene mediates insensitivity to SAMs. To this end, the *Asc-1* and *asc1* genes were overexpressed in tomato hairy roots, and the effects of SAMs were determined. The recent identification of the SAM-sensitive and *A. alternata* f. sp. *lycopersici*-susceptible *Nicotiana umbratica* (Brandwagt et al. 2001) further facilitated studying the function of *Asc-1* in transgenic plants. We show that overexpression of *Asc-1*, but not *asc-1*, mediates an increased insensitivity to SAMs and confers resistance to the SAM-producing phytopathogenic fungus *A. alternata* f. sp. *lycopersici*.

Fig. 1. Phenotypes of tomato hairy root transformants (over)expressing *Asc-1* or *asc-1* grown on root medium supplied with AAL toxins. **A**, Phenotypes of reference hairy root transformants with a single-copy binary vector, pGreen029 or pBAsc404 (with *Asc-1* driven by its endogenous promoter) (Brandwagt et al. 2000). The genotypes of the recipient tomato *Asc* isogenic lines are indicated on the top. Discriminating concentrations of AAL toxins are indicated on the left. Necrosis of the root tip (arrows) was only observed for transformants of the *asc,asc* recipient line. All hairy root transformants grown on toxin-free medium have a similar phenotype as the *Asc,Asc* pGreen029 transformants grown on 0.004 μ M AAL toxins and are not shown. Scale bar is 1 cm. **B through E**, Phenotypes of representative hairy root transformants that overexpress *Asc-1* or *asc-1* in certain recipient tomato *Asc* isogenic lines. **B and C**, e35S-*asc-1* in the *Asc,Asc* genotype at **B**, 0.04 μ M AAL toxins and **C**, 0.4 μ M AAL toxins; **D and E**, e35S-*Asc-1* in the *asc,asc* genotype at 0.4 μ M AAL toxins. The phenotypes of the e35S-*asc-1* (data not shown) and pGreen029 transformants in the *asc,asc* genotype were similar.

RESULTS

Agrobacterium rhizogenes-induced transgenic hairy roots provided an efficient system to determine possible effects of overexpression of the *Asc-1* and *asc-1* genes on SAM (in)sensitivity. The overexpression of the *asc-1* pseudogene was included in this study to exclude the possibility that overexpression of an *Asc* mRNA would lead to artifacts related to transgene-induced gene silencing of the endogenous gene homologs and to determine if overexpression of *asc-1* would suppress SAM insensitivity. Constructs in which the *Asc-1* and *asc-1* genes were driven by an enhanced *Cauliflower mosaic virus* e35S promoter were produced (e35S-*asc-1* and e35S-*Asc-1*, respectively). These constructs were subsequently used to generate transgenic hairy roots of recipient tomato *Asc* isogenic lines. As reference transformants, pGreen029 transformants of the *Asc,asc* and *Asc,Asc* recipient tomato lines were made in addition to the published pGreen029 and pBasc404 hairy root transformants in the *asc,asc* recipient tomato line (Brandwagt et al. 2000). The effects of the (over)expression of the *Asc* genes were assessed by growing the reference and overexpression hairy root transformants on root medium containing a series of AAL toxins. Following incubation, the effects were determined in two ways. First, the visual phenotypes of the reference hairy root transformants (Fig. 1A) and the overexpression transformants (Fig. 1B to E) were compared. Second, the growth rate inhibition of the reference hairy root transformants (Fig. 2A) and overexpression transformants (Fig. 2B) were determined relative to the growth rate on toxin-free root medium. On toxin-free medium, no obvious phenotype was observed in the e35S-*asc-1* or e35S-*Asc-1* hairy root transformants. The presence of the e35S-*asc-1* construct in either the *asc,asc* background or the *Asc,Asc* background did not lead to enhanced susceptibility to AAL toxins. The observed hairy root phenotypes at all toxin concentrations were similar to the control lines with respect to root tip necrosis (Fig. 1). Moreover, by comparison of Figure 2A with B, it became clear that the AAL toxin-induced growth inhibition of the 35S-*asc-1* lines in both *asc,asc* and *Asc,Asc* recipient tomato lines was similar to the corresponding control lines. Effects on toxin insensitivity were only observed for hairy root transformants that overexpressed *Asc-1* in an *asc,asc* background. The hairy

root phenotype of the e35S-*Asc-1* transformants at low toxin concentrations and at 0.4 μ M AAL toxins was similar (Fig. 1D and E). Moreover, the root growth of these transformants was uninhibited at 0.4 μ M AAL toxins (Fig. 2B).

The reference hairy root transformants with a single insertion of the *Asc-1* gene driven by its endogenous promoter tested equally as sensitive as the hairy root transformants produced from heterozygous *Asc,asc* plants on the basis of the observed phenotype (Fig. 1A) and root growth inhibition (Fig. 2A). This suggested a dose dependency of *Asc-1* in relation to toxin insensitivity. Therefore, we looked for a correlation between overexpression level and AAL-toxin insensitivity. All e35S-*Asc-1* and 35S-*asc-1* hairy root transformants showed detectable, but not identical, expression levels of *Asc* (Fig. 3). The mean overexpression level of e35S-*asc-1* transformants was an estimated 12-fold less than the mean overexpression level of e35S-*Asc-1* transformants (Fig. 3), suggesting a rapid turnover of the mRNA as a result of the mutations present in *asc-1*. No clear correlation between the overexpression level of the e35S-*Asc-1* transformants and the level of SAM insensitivity was found (Fig. 3), suggesting that the observed levels of overexpression were sufficient to mediate the high-insensitivity phenotype.

The high-insensitivity phenotype of the 35S-*Asc-1* tomato hairy roots prompted us to study the effects of overexpression of the *Asc* genes in whole plants. For instance, it could be determined if overexpression of *Asc-1* (in an *rr* background) would mediate enhanced SAM insensitivity in germinating seedlings and leaves that display maximum SAM sensitivity in the case of wild-type tomato and *N. umbratica*. Moreover, it would indicate if overexpression of *Asc-1* mediates resistance to *A. alternata* f. sp. *lycopersici*. *N. umbratica* could be efficiently transformed using *Agrobacterium tumefaciens*-mediated gene transfer (data not shown) and was tested susceptible to *A. alternata* f. sp. *lycopersici* previously (Brandwagt et al. 2001). Therefore, *N. umbratica* was chosen as an alternative to *L. esculentum* to study the effects of overexpression of the *Asc-1* gene in planta. The constructs pGreen0029, pBasc501 (e35S-*Asc-1*), and pBasc634 (e35S-*asc-1*) were transformed to an *rr* (equivalent to *asc,asc*) *N. umbratica* genotype. Single-T-DNA copy diploid primary transgenic plants (T_1) were selected for further analysis (Table 1). The self-progeny of these plants

Fig. 2. Root growth inhibition by AAL toxins in control e35S-*asc-1* or e35S-*Asc-1* tomato hairy root transformants. The root growth inhibition was determined relative to the growth on control root medium without toxin. The recipient tomato *Asc* genotypes and the constructs used for transformation are indicated on the top. The mean value of five independent transformants for each construct-recipient genotype combination is presented. The corresponding standard deviation is indicated by bars. **A**, Root growth inhibition observed in control experiments. pGreen029 is the binary vector used to produce both pBasc404 (*Asc-1* driven by its endogenous tomato promoter) and the e35S vectors. **B**, Root growth inhibition observed in transformants that overexpress *Asc-1* or *asc-1*. The e35S-*asc-1* transformants tested similarly sensitive to AAL toxins compared with the corresponding control pGreen029 transformants. The e35S-*Asc-1* transformants tested more insensitive to AAL toxins than did the *Asc,Asc* pGreen029 control transformants.

(T₂) segregated 3:1 for either insensitivity to kanamycin, 0.2 μ M AAL toxins, or both, in the germination and detached leaf assays, assuming a 95% confidence limit in the χ^2 test. Subsequently, the T₂ seeds were germinated on a series of FB₁ and AAL-toxin concentrations. Even at the highest concentrations of SAMs, i.e., 100 μ M, a 3:1 segregation for toxin insensitivity was observed in germinating seeds of the e35S-*Asc-1* *N. umbratica* lines (Table 1). The growth of approximately 75% of the T₂ seedlings of the e35S-*Asc-1* lines was similar to that of the seedlings in the water control incubations. In contrast, seedlings of the control *rr* and *RR* lines were completely necrotic or displayed severe growth inhibition of roots and (hypo)cotyledons at high SAM concentrations (Table 1). The segregation ratios and phenotype at high SAM concentrations suggested that 35S-*Asc-1* could also mediate increased insensitivity in *N. umbratica* plants. To find out if a high *Asc-1* expression would lead to increased insensitivity in mature plants, the wild-type *rr* *N. umbratica* parent line, T₂ plants of five T₁ lines, and the wild-type *RR* *N. umbratica* control line were grown in the greenhouse for expression analysis, leaf bioassays, and fungal inoculations. The plants were first analyzed by RNA blots to determine the level of *Asc-1* or *asc-1* overexpression in each plant (Fig. 4A). The approximate 14-fold lower expression in the progenies of the e35S-*asc-1* transgenic *N. umbratica* plants, G69171 and G69173, relative to progenies of the e35S-*Asc-1* plants, G69612, G69163, and G69203 (Fig. 4), paralleled the differences observed in tomato hairy roots (Fig. 3). The tomato *Asc-1* pre-mRNA is spliced correctly in *N. umbratica*, confirmed by the estimated size of the mature mRNA on blot (Fig. 4A). The *asc-1* mRNA, however, runs at a slightly higher molecular weight in two primary transformants, possibly by incomplete pre-mRNA splicing. The *N. umbratica* plants that overexpressed *Asc-1* or *asc-1* showed no obvious phenotype under standard greenhouse conditions. In all plants tested, the overexpression of *Asc-1*, but not *asc-1*, cosegregated with insensitivity of detached leaves to 10 μ M AAL toxins or 100 μ M FB₁ in the leaf bioassay. At these concentrations,

Fig. 3. Relative expression levels of e35S-*asc-1* and e35S-*Asc-1* constructs in hairy root transformants in relation to root growth inhibition by AAL toxins. The mean root growth of the five independent e35S-*asc-1* and e35S-*Asc-1* hairy root transformants at 0.4 μ M AAL toxins was plotted against their expression levels relative to glyceraldehyde phosphate dehydrogenase (GAPDH). The e35S-*asc-1* hairy root transformants showed a 2 to 19% overexpression level in both *asc,asc* and *Asc,Asc* parent lines. The overexpression level of the e35S-*Asc-1* hairy root transformants in the *asc,asc* parent line ranged from 35 to 286% overexpression relative to GAPDH. Although the relative expression levels of the individual e35S-transformants varied, their root growth inhibition at 0.4 μ M AAL toxins was similar.

leaves of the control *rr* and *RR* plants were necrotic (Table 1). From the above segregation analyses, it could be concluded that overexpression of *Asc-1*, but not *asc-1*, mediates enhanced insensitivity to FB₁ and AAL toxins in *N. umbratica* plants. Whether the overexpression of *Asc-1* in *N. umbratica* plants mediated resistance to *A. alternata* f. sp. *lycopersici* remained elusive. Therefore, the segregating T₂ populations were assayed for their susceptibility to *A. alternata* f. sp. *lycopersici* spore inoculation, using wild-type *N. umbratica* *rr* and *RR* plants as controls. Resistant T₂ plants displayed no necrosis, whereas susceptible T₂ plants completely wilted, as observed previously (Brandwagt et al. 2001). The symptoms of resistant and susceptible phenotypes of the T₂ plants were indistinguishable from those of the *RR* and *rr* control plants (Fig. 4B). An absolute cosegregation of the overexpression of *Asc-1*, a high insensitivity to SAMs, and resistance to *A. alternata* f. sp. *lycopersici* was observed in T₂ plants of the 35S-*Asc-1* lines tested (Fig. 4A and B). This unequivocally demonstrated that the *Asc-1* gene, besides mediating SAM insensitivity, also provides resistance to *A. alternata* f. sp. *lycopersici* upon overexpression in an alternative host species.

DISCUSSION

In all plant species tested, insensitivity to SAMs coincides with resistance to *A. alternata* f. sp. *lycopersici* (Brandwagt et al. 2001). The overexpression of *Asc-1* in *N. umbratica* plants mediated resistance to the fungus, as expected, and mediated enhanced insensitivity to SAMs in tomato hairy roots and *N. umbratica* germinating seedlings and detached leaves. In *Lycopersicon* species, variation in SAM sensitivity has been observed. For instance, leaves of *Asc,Asc* *L. esculentum* genotypes show a complete necrosis after incubation in 40 μ M AAL toxins. Seeds and leaves of *L. pennellii* and *L. peruvianum* are insensitive to high concentrations of AAL toxins (up to 100 μ M) and are resistant to *A. alternata* f. sp. *lycopersici* infection (van der Biezen et al. 1995). The high-insensitivity locus in *L. pennellii* has been mapped at the *L. esculentum* *Asc* locus and is inherited as a single-Mendelian, and completely dominant, trait (van der Biezen et al. 1995). Self-progeny of hemizygous e35S-*Asc-1* *N. umbratica* plants showed a 3:1 segregation at high SAM concentrations in germination and detached-leaf bioassays (Table 1). Thus, overexpression from a single T-DNA is sufficient to mediate a high-insensitivity phenotype, exemplified by the similar *Asc-1* overexpression levels of SAM-insensitive plants in the segregating progenies (Fig. 4A). Together, the above data suggest that a high natural level of expression of *Asc-1* homologs could be present in plant species highly insensitive to SAMs (Abbas et al. 1995; van der Biezen et al. 1995). The relative expression levels of *Asc-1* homologs may also explain the differences in SAM sensitivity found between different plant tissues of the *Asc* isolines (Brandwagt et al. 1998) and between mammalian organs and their corresponding cultured (cancer) cell lines (Shier et al. 1991; Vesonder et al. 1993; Wang et al. 1996a).

The abundance of the wild-type *Asc-1* mRNA has been estimated to be a few copies per cell (Brandwagt et al. 2000). Tomato hairy roots (Fig. 1A) and tomato and *N. umbratica* plants heterozygous for the *Asc* locus are intermediately sensitive to SAMs (Brandwagt et al. 2001). These data suggest that *Asc-1* is not transcriptionally regulated. The moderate expression levels (Figs. 3 and 4A) were sufficient to confer a high-insensitivity phenotype in tomato hairy roots and *N. umbratica* plants. Yeast is an example of the effects of increased overexpression of LAG1-like genes in another species. In yeast, increased expression of *Lag1* from a galactose-inducible promoter results in the senescence of many young cells, but those that survive of-

ten live longer than wild-type cells. Under moderate levels of expression, the life span of the parent strain is increased by at least 60% (Jazwinski 1993). Plants have a characteristic life span of development and photosynthetic productivity, after which the senescence cell death program is triggered (Bleecker and Patterson 1997; Pennell and Lamb 1997). Senescence can thus be considered an indicator of life span in plants. Besides age, senescence can be triggered by external factors such as phytohormone application, continuous darkness, temperature, and pathogen attack (Quirino et al. 2000). The e35S-*Asc-1* overexpression lines provide a tool to study the possible function of *Asc-1* in plant longevity. In addition to increased insensitivity to SAMs, these lines may also show enhanced resistance to induction of senescence.

Apart from a putative effect of *Asc-1* on longevity, by which mechanism would the increased expression of the *Asc-1* gene explain the high-insensitivity phenotype? Inhibition of SAT in eukaryotes generally leads to the accumulation of free sphingoid bases and their phosphorylated forms, followed by a depletion of complex ceramides. Consequently, overexpression of *Asc-1* may provide increased insensitivity to the elevated concentrations of free (phosphorylated) sphingoid bases or to the depletion of complex ceramides (Brandwagt et al. 2000). The unraveling of enzymatic pathways and functions of sphingolipids is most advanced in yeast (Dickson and Lester 1999) and may indicate which of the above hypotheses is most likely. The yeast LAG1 and LAC1 proteins were recently shown to facilitate acyl-CoA-dependent ceramide synthesis (Guillas et al. 2001). The *Asc-1* gene is homologous to LAG1 and LAC1 (Brandwagt et al. 2000). Therefore, overexpression of *Asc-1* could increase the production of (suppressor) ceramides and thereby enhance the neutralization of the toxic effects of SAMs on ceramide biosynthesis (Brandwagt et al. 2000). Besides the visible effects of the SAMs on leaf necrosis and seedling development described here, it becomes necessary to understand the biochemical function of the ASC1 protein in the mechanism of insensitivity to SAMs. For instance, the sphingolipid composition and concentration in leaves of SAM-insensitive and SAM-sensitive plant genotypes may be determined before and after inhibitor treatments (Sullards 2000). In conclusion, the *Asc-1* gene mediates resistance to *A. alternata* f. sp. *lycopersici* in the alternative host *N. umbratica*. Upon overexpression, *Asc-1* confers enhanced insensitivity to SAMs in plants. To study the role of the *Asc-1* gene in plant sphingolipid me-

tabolism, which may regulate plant cell homeostasis, senescence, and endocytosis of GPI-anchored proteins, will be an intriguing subject.

MATERIALS AND METHODS

Plant materials.

The *L. esculentum* homozygous SAM-insensitive (*Asc,Asc*) or SAM-sensitive (*asc,asc*) near-isogenic *Asc* lines were used (Clouse and Gilchrist 1987). Heterozygous *Asc,asc* seeds for hairy root transformation were produced by standard emasculation and pollination techniques. *N. umbratica* (PI271993) seeds came from the Botanical Garden of the University of Nijmegen (Nijmegen, The Netherlands). This *N. umbratica* accession contains a single-Mendelian locus analogous to the tomato *Asc* locus. Self-progeny of lines previously determined to be homozygous SAM insensitive (*RR*) or SAM-sensitive (*rr*) were used (Brandwagt et al. 2001). All plants were grown under standard Dutch greenhouse conditions (20°C, 60% humidity, and 15 kilolux light intensity maintained by automated artificial lights).

Sphinganine analog mycotoxins.

FB₁ (1 mM) (Sigma, St. Louis, MO, U.S.A.) was stored in water at -20°C. A 95:5% mixture of the equally phytotoxic AAL toxins T_A and T_B (10 mM) was isolated and quantified as described by Brandwagt and associates (2001).

T-DNA constructs.

Standard molecular biological techniques were used (Sambrook et al. 1989). All T-DNA constructs were made with the pGreen029 binary vector (Hellens et al. 2000). In addition, because it was not known if the endogenous tomato *Asc-1* promoter would be functional in this species, the e35S promoter was used for functional studies in *N. umbratica* plants. The enhanced (e)35S-*Asc-1* and e35S-*asc-1* binary overexpression constructs were generated as follows. The Asp718 (*KpnI*/*XbaI*) polylinker from pGreen029 SK LR (Hellens et al. 2000) was removed by digestion, and the sticky ends of the backbone were filled in and religated, resulting in pGreen0K. The *NorI* fragment of a plant overexpression cassette with a *Cauliflower mosaic virus* 35S promoter with a duplication of the enhancer region (e35S) and a nopaline synthase terminator, (Tnos) *SacI* polylinker of pGreen0K. The resulting pGreen1K contains

Table 1. Segregation of insensitivity to sphinganine-analog mycotoxins and resistance to *Alternaria alternata* f. sp. *lycopersici* in progeny of *Nicotiana umbratica* T₁ lines

<i>N. umbratica</i> line	T-DNA construct	In vitro germination bioassays						Plant bioassays	
		50 mg of kanamycin per liter ^a	0.2 µM AAL toxins per liter ^b	100 µM FB ₁ ^b	100 µM AAL toxins ^b	10 µM AAL toxins ^c	<i>A. alternata</i> f. sp. <i>lycopersici</i> ^d	Detached leaves	Fungal inoculation
wild type <i>rr</i>	none	0:42	0:43	0:50	0:47	0:12	0:12		
wild type <i>RR</i>	none	0:34	43:0	0:40 ^e	0:32 ^e	0:11	11:0		
G69178	pGreen029	25:11	0:78	0:56	0:43	0:8	0:8		
G69171	pBasc634 (e35S- <i>asc-1</i>)	31:13	0:38	0:50	0:43	0:12	0:12		
G69173	pBasc634 (e35S- <i>asc-1</i>)	35:9	0:63	0:38	0:67	0:12	0:12		
G69162	pBasc501 (e35S- <i>Asc-1</i>)	28:9	54:18	45:19	50:15	10:4	10:4		
G69163	pBasc501 (e35S- <i>Asc-1</i>)	34:10	69:26	67:21	38:14	14:6	14:6		
G69203	pBasc501 (e35S- <i>Asc-1</i>)	31:9	30:11	36:16	37:14	9:3	9:3		

^a Segregation ratios are shown as kanamycin-insensitive/kanamycin-sensitive seedlings.

^b Segregation ratios are shown as toxin-insensitive/toxin-sensitive seedlings.

^c Segregation ratios are shown as toxin-insensitive/toxin-sensitive detached leaves in the detached leaf bioassay.

^d Segregation ratios are shown as fungus-resistant/fungus-susceptible plants upon inoculation with fungal isolate VU2001.

^e Growth inhibition observed but no necrosis of the root tip.

a polylinker with the following unique restriction sites: (e35S)-*Clal-XbaI-EcoRI-BamHI*-(Tnos). Due to the low expression of *Asc-1* in tomato, an *Asc-1* cDNA was not available until recently. The *Asc-1* and *asc-1* open reading frames were therefore amplified with oligonucleotides BASC86 (*BamHI*, reverse) CGGGATCCCGATCAGTCTTTGTGGTCATCATC and BASC87 (*EcoRI*, forward) GGAATTCCTGCAATTCATTGAACTACAAC (Brandwagt et al. 2000) by *Pfu* DNA-poly-

merase from 100 ng of genomic DNA of the corresponding *Asc* isogenic tomato lines, according to the manufacturer's instructions (Stratagene, La Jolla, CA, U.S.A.). The polymerase chain reaction (PCR) products were purified from DNA gel by the Qiaex procedure (Qiagen, Chatsworth, CA, U.S.A.) and cloned into pGEM-Teasy (Promega, Madison, WI, U.S.A.). Subsequently, the resulting plasmids were cloned as *BamHI/EcoRI* fragments in pGreen1K. The inserts were sequenced and contained no mutations relative to the wild-type sequences.

Plant transformation and selection.

Two selected constructs, pBAsc501 (e35S-*Asc-1*) and pBAsc634 (e35S-*asc-1*), and the control, T-DNA pGreen029, were electroporated into *Agrobacterium tumefaciens* MOG301 and *Agrobacterium rhizogenes* LBA9402 containing the helper plasmid pSoup (Hellens et al. 2000). Tomato hairy roots of the *Asc* isogenic lines were produced on kanamycin selection root medium as described by Brandwagt and associates (2000). Transgenic *asc,asc* hairy roots, containing a single-copy pBAsc404 construct with the *Asc-1* gene driven by its endogenous promoter, were obtained previously (Brandwagt et al. 2000). Isolation of genomic DNA and subsequent PCR analysis of transgenic hairy roots and *N. umbratica* plants were performed as described by van der Biezen and associates (1996). Genomic DNA of hairy roots was further column-purified (Qiagen) to remove contaminant polysaccharides. The presence of the NPTII transgenes was determined by PCR amplification with the oligonucleotides NPT-R 5'-AGAAGAACTGGTCAAGAAGG-3' and NPT-F 5'-CCGGTTCTTTTGTCAA-GAC-3'. The presence of the e35S-*Asc-1* or e35S-*asc-1* transgenes was determined with the oligonucleotides BASC8 5'-CACTTCCGTCATGAAGTGC-3' and BASC53 5'-CTACTTGCACCTTCTGTGAC-3'. In addition, Southern blot analysis (van der Biezen et al. 1996) by hybridization with the NPT-F/R and BASC-8/53 PCR fragments verified the copy number of the transgenes in the hairy roots.

Transgenic *N. umbratica* plants were produced by a leaf-disk transformation procedure and kanamycin selection (Horsch et al. 1985). Twenty e35S-*Asc-1*, fifteen e35S-*asc-1*, and fourteen pGreen029 primary *N. umbratica* transformants were selected by PCR with the above NPT and BASC8/53 oligonucleotides. To circumvent T-DNA position effects, diploid single-T-DNA copy T₁ plants were selected on the basis of normal stomata and a 3:1 segregation of resistance to kanamycin (for e35S-*asc-1*) or AAL toxins (for e35S-*Asc-1*) of the self-progeny in the germination assay.

RNA analysis.

Total RNA of hairy roots and *N. umbratica* leaves was isolated as described (van der Biezen et al. 1996). Glyoxal-denatured RNA (10 µg) was separated on Bis-Tris/PIPES (piperazine-*N,N'*-bis[2ethanesulfonic acid]) agarose gels (Burnett 1997) and transferred to Hybond-N⁺ nylon membranes (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) by overnight capillary blotting with 20 mM NaOH (Sambrook et al. 1989). The filters were subsequently neutralized in 0.5 M Tris-HCL, pH 7.5, and the RNA was immobilized by UV crosslinking. The *Asc-1* probe was prepared by PCR with digoxigenin-labeled alkali-labile dUTP (Roche Molecular Biochemicals, Indianapolis, IN, U.S.A.) and oligonucleotides BASC86 and BASC87, using pBAsc404 as template DNA (Brandwagt et al. 2000). Hybridization of the filters was performed in modified Church and Gilbert buffer (1% [wt/vol] defatted milk powder; 1 mM EDTA; 0.5 M NaHPO₄, pH 7.2; 10% [wt/vol] sodium dodecyl sulfate [SDS]) at 65°C for 16 h. The filters were washed with 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.1% (wt/vol)

Fig. 4. Overexpression of *Asc-1* mediates resistance to *Alternaria alternata* f. sp. *lycopersici* in *Nicotiana umbratica* plants. **A**, RNA blots hybridized with the tomato *Asc-1* gene and washed at high stringency to analyze the expression of possible homologs in the *N. umbratica* parent line (S), *asc-1* in two representative single-copy e35S-*asc-1* independent primary transformants of the parent line (G69171 and G69173), and *Asc-1* in self-progeny of the selected independent single-copy e35S-*Asc-1* transformants, G69162, G69163, and G69203. As a control for proper loading, the ribosomal RNA fragments on the corresponding ethidium bromide-stained agarose gels are shown. **B**, *A. alternata* f. sp. *lycopersici* inoculation of two representative plants of the G69163 self-progeny that segregated for overexpression of e35S-*Asc-1* (marked with an asterisk in **A**). Approximately 75% of the progeny tested insensitive to high sphinganine-analog mycotoxin (SAM) concentrations and resistant to the fungus (R), and 25% tested SAM sensitive and susceptible to *A. alternata* f. sp. *lycopersici* (S).

SDS at 65°C. Detection was performed on Kodak X-Omat AR films (Eastman-Kodak, Rochester, NY, U.S.A.) for 5 min as described by Burnett (1997).

Tomato hairy root growth bioassay.

Five kanamycin-resistant primary transformants per construct were randomly selected and further subcultured on kanamycin root medium. Terminal root segments of approximately 1 cm were excised and transferred to root medium supplemented with purified AAL toxins. The position of the root tip was marked. After 1 week of incubation in a climate cabin (22°C at 3 kilolux light intensity), the root length was measured from the marked point with a precision slide ruler. For each transformant, 28 root segments were measured to minimize the standard deviation. The root growth of each transformant on control root medium without toxin was set to 100%.

Determination of *Asc* expression levels.

Each of the five hairy root transformants per construct was subcultured on three plates with kanamycin root medium for 3 weeks in a climate cabin (22°C at 3 kilolux light intensity), and its RNA was isolated and analyzed as described above. The expression of *Asc-1* driven by its endogenous tomato promoter was not detectable on RNA blots. Therefore, the transcription level of the glyceraldehydephosphate dehydrogenase (GAPDH) gene was used as a standard in the estimation of the *Asc-1* and *asc-1* overexpression levels. The overexpression of *Asc-1* or *asc-1* did not influence the expression of GAPDH relative to the pGreen029 controls. Expression levels were quantified by comparing band intensities of the three RNA preparations to background noise with Imagequant Software according to the manufacturer's manual (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

In vitro germination bioassays.

Seeds were germinated in a climate cabin (22°C at 3 kilolux light intensity) in sealed 9-cm petri dishes on filter papers (#595; Schleicher & Schuell, Inc., Dassel, Germany) soaked in 4 mL of 0.1 µM gibberellic acid-3 (GA₃) supplemented with logarithmic dilutions of FB₁ (0 and 1 to 100 µM), purified AAL toxins (0 and 0.3 to 100 µM), or 50 mg of kanamycin per liter. Symptoms were scored 10 days after sowing, when either the elongating roots of toxin-resistant seedlings had root hairs and the root tips of sensitive seedlings showed a brown necrosis for the SAM incubations or the cotyledons of kanamycin-sensitive *rr* wild-type seeds were bleached for the kanamycin incubations.

Detached leaf bioassay.

Leaf bioassays were performed in triplicate, according to Gilchrist and associates (1992), by incubation of detached leaflets of 6-week-old plants in 9-cm petri dishes on Schleicher & Schuell filter papers soaked with 3 ml of the 0.2-µM-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 at 3 kilolux light intensity) to minimize variation.

A. alternata f. sp. *lycopersici* assay.

The fungal inoculation was performed essentially as described by Brandwagt and associates (2001). Briefly, *A. alternata* f. sp. *lycopersici* was grown for 14 days on V8 plates (20% [vol/vol] V8 juice [Campbell Soup Co., Camden, NJ, U.S.A.], 30 mM CaCO₃, and 1.6% agar) at 25°C under continuous near-UV light. Spores were suspended in 0.05% (vol/vol) Tween 80 in water, filtered, washed twice in tap water to remove in vitro-produced AAL toxins, and diluted to a final con-

centration of 10⁶ spores per ml (Grogan et al. 1975). Six-week-old *N. umbratica* plants were covered by a plastic tunnel and preincubated until humidity reached 100%. Inoculation was performed with 10 ml of inoculum per plant and 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 *rr* control *N. umbratica* plants were completely wilted.

ACKNOWLEDGMENTS

The authors thank M. Kuipers for greenhouse assistance, K. Spelt for constructing pGreen1K, G. van der Weerden for *Nicotiana* seeds, and D. Gilchrist for the tomato *Asc* lines and the *Alternaria alternata* isolate. B. F. Brandwagt was sponsored by the Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO) and by the Associatie van Biotechnologische Onderderzoeksscholen Nederland (ABON).

LITERATURE CITED

- Abbas, H. K., Tanaka, T., Duke, S. O., Porter, J. K., Wray, E. M., Hodges, L., Sessions, A. E., Wang, E., Merrill, A. H., and Riley, R. T. 1994. Fumonisin- and AAL toxin-induced disruption of sphingolipid metabolism with accumulation of free sphingoid bases. *Plant Physiol.* 106:1085-1093.
- Abbas, H. K., Tanaka, T., Duke, S. O., and Boyette, C. D. 1995. Susceptibility of various crop and weed species to AAL toxin, a natural herbicide. *Weed Technol.* 9:125-130.
- Akamatsu, H., Itoh, Y., Kodama, M., Otani, H., and Kohmoto, K. 1997. AAL-toxin-deficient mutants of *Alternaria alternata* tomato pathotype by restriction enzyme-mediated integration. *Phytopathology* 87:967-972.
- The Arabidopsis Genome Initiative 2000. Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature* 408:796-815.
- Barz, W. P., and Walter, P. 1999. Two endoplasmic reticulum (ER) membrane proteins that facilitate ER-to-Golgi transport of glycosylphosphatidylinositol-anchored proteins. *Mol. Biol. Cell* 10:1043-1059.
- Bleecker, A. B., and Patterson, S. E. 1997. Last exit: Senescence, abscission, and meristem arrest in *Arabidopsis*. *Plant Cell* 9:1169-1179.
- Brandwagt, B. F., Mesbah, L. A., Laurent, P. L., Takken, F. L. W., Kneppers, T. J. A., Nijkamp, H. J. J., and Hille, J. 1998. The interaction of *Alternaria alternata* f. sp. *lycopersici* and its AAL toxins with tomato. Pages 317-330 in: *Molecular Genetics of Host-Specific Toxins in Plant Disease*. K. Kohmoto, and O. C. Yoder (eds). Kluwer, Dordrecht, The Netherlands.
- Brandwagt, B. F., Mesbah, L. A., Takken, F. L. W., Laurent, P. L., Kneppers, T. J. A., Hille, J., and Nijkamp, H. J. J. 2000. A longevity assurance gene homolog of tomato mediates resistance to *Alternaria alternata* f. sp. *lycopersici* toxins and fumonisin B₁. *Proc. Natl. Acad. Sci. U.S.A.* 97:4961-4966.
- Brandwagt, B. F., Kneppers, T. J. A., van der Weerden, G. M., Nijkamp, H. J. J., and Hille, J. 2001. Most AAL toxin-sensitive *Nicotiana* species are resistant to the tomato fungal pathogen *Alternaria alternata* f. sp. *lycopersici*. *Mol. Plant-Microbe Interact.* 14:460-470.
- Burnett, W. V. 1997. Northern blotting of RNA denatured in glyoxal without buffer recirculation. *Biotechniques* 22:668-671.
- Clouse, S. D., and Gilchrist, D. G. 1987. Interaction of the *asc* locus in *F₈* paired lines of tomato with *Alternaria alternata* f. sp. *lycopersici* and AAL-toxin. *Phytopathology* 77:80-82.
- Desjardins, A. E., and Hohn, T. M. 1997. Mycotoxins in plant pathogenesis. *Mol. Plant-Microbe Interact.* 10:147-152.
- Desjardins, A. E., and Plattner, R. D. 2000. Fumonisin B(1)-nonproducing strains of *Fusarium verticillioides* cause maize (*Zea mays*) ear infection and ear rot. *J. Agric. Food Chem.* 48:5773-5780.
- Dickson, R. C., and Lester, R. L. 1999. Metabolism and selected functions of sphingolipids in the yeast *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta* 1438:305-321.
- D'Mello, N. P., Childress, A. M., Franklin, D. S., Kale, S. P., Pinswasdi, C., and Jazwinski, S. M. 1994. Cloning and characterization of LAG1, a longevity-assurance gene in yeast (published erratum appears in *J. Biol. Chem.* 1994. 269:28522). *J. Biol. Chem.* 269:15451-15459.
- Gilchrist, D. G., Ward, B., Moussato, V., and Mirocha, C. J. 1992. Genetic and physiological response to fumonisin and AAL-toxin by intact tissue of a higher plant. *Mycopathologia* 117:57-64.
- Grogan, R. G., Kimble, K. A., and Misaghi, I. 1975. A stem canker dis-

- ease of tomato caused by *Alternaria alternata* f. sp. *lycopersici*. *Phytopathology* 65:880-886.
- Guillas, I., Kirchman, P. A., Chuard, R., Pfefferli, M., Jiang, J. C., Jazwinski, S. M., and Conzelmann, A. 2001. C26-CoA-dependent ceramide synthesis of *Saccharomyces cerevisiae* is operated by Lag1p and Lac1p. *EMBO (Eur. Mol. Bio. Organ.) J.* 20:2655-2665.
- Hellens, R. P., Edwards, E. A., Leyland, N. R., Bean, S., and Mullineaux, P. M. 2000. pGreen: A versatile and flexible binary *Ti* vector for *Agrobacterium*-mediated plant transformation. *Plant Mol. Biol.* 42:819-832.
- Horsch, R. B., Fry, J. E., Hoffman, N. L. D. E., Rogers, S. G., and Fraley, R. T. 1985. A simple and general method for transferring genes into plants. *Science* 227:1229-1231.
- Jazwinski, S. M. 1993. The genetics of aging in the yeast *Saccharomyces cerevisiae*. *Genetica* 91:35-51.
- Jiang, J. C., Kirchman, P. A., Zagulski, M., Hunt, J., and Jazwinski, S. M. 1998. Homologs of the yeast longevity gene LAG1 in *Caenorhabditis elegans* and human. *Genome Res.* 8:1259-1272.
- Lynch, D. V. 2000. Enzymes of sphingolipid metabolism in plants. *Methods Enzymol.* 311:130-149.
- Merrill, A. H., Liotta, D. C., and Riley, R. T. 1996. Fumonisin: Fungal toxins that shed light on sphingolipid function. *Trends Cell Biol.* 6:218-223.
- Merrill, A. H., Jr., Schmelz, E. M., Dillehay, D. L., Spiegel, S., Shayman, J. A., Schroeder, J. J., Riley, R. T., Voss, K. A., and Wang, E. 1997. Sphingolipids—The enigmatic lipid class: Biochemistry, physiology, and pathophysiology. *Toxicol. Appl. Pharmacol.* 142:208-225.
- Mesbah, L. M., van der Weerden, G. M., Nijkamp, H. J. J., and Hille, J. 2000. Sensitivity among species of the *Solanaceae* to AAL toxins produced by *Alternaria alternata* f. sp. *lycopersici*. *Plant Pathol.* 49:734-741.
- Muniz, M., Morsomme, P., and Riezman, H. 2001. Protein sorting upon exit from the endoplasmic reticulum. *Cell* 104:313-320.
- Norred, W. P., Bacon, C. W., Riley, R. T., Voss, K. A., and Meredith, F. I. 1999. Screening of fungal species for fumonisin production and fumonisin-like disruption of sphingolipid biosynthesis. *Mycopathologia* 146:91-98.
- Pennell, R. I., and Lamb, C. 1997. Programmed cell death in plants. *Plant Cell* 9:1157-1168.
- Quirino, B. F., Noh, Y. S., Himelblau, E., and Amasino, R. M. 2000. Molecular aspects of leaf senescence. *Trends Plant Sci.* 5:278-282.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U.S.A.
- Shier, W. T., Abbas, H. K., and Mirocha, C. J. 1991. Toxicity of the mycotoxins fumonisins B₁ and B₂ and *Alternaria alternata* f. sp. *lycopersici* toxin (AAL) in cultured mammalian cells. *Mycopathologia* 116:97-104.
- Skrzypek, M., Lester, R. L., and Dickson, R. C. 1997. Suppressor gene analysis reveals an essential role for sphingolipids in transport of glycosylphosphatidylinositol-anchored proteins in *Saccharomyces cerevisiae*. *J. Bacteriol.* 179:1513-1520.
- Stone, J. M., Heard, J. E., Asai, T., and Ausubel, F. M. 2000. Simulation of fungal-mediated cell death by fumonisin B₁ and selection of fumonisin B₁-resistant (fbr) *Arabidopsis* mutants. *Plant Cell* 12:1811-1822.
- Sullards, M. C. 2000. Analysis of sphingomyelin, glucosylceramide, ceramide, sphingosine, and sphingosine 1-phosphate by tandem mass spectrometry. *Methods Enzymol.* 312:32-45.
- van der Biezen, E. A., Glagotskaya, T., Overduin, B., Nijkamp, H. J. J., and Hille, J. 1995. Inheritance and genetic mapping of resistance to *Alternaria alternata* f. sp. *lycopersici* in *Lycopersicon pennellii*. *Mol. Gen. Genet.* 247:453-461.
- van der Biezen, E. A., Brandwagt, B. F., van Leeuwen, W., Nijkamp, H. J. J., and Hille, J. 1996. Identification and isolation of the FEEBLY gene from tomato by transposon tagging. *Mol. Gen. Genet.* 251:267-280.
- Vesonder, R. F., Peterson, R. E., Labeda, D., and Abbas, H. K. 1992. Comparative phytotoxicity of the fumonisins, AAL toxin, and yeast sphingolipids in *Lemna minor* L. (duckweed). *Arch. Environ. Contam. Toxicol.* 23:464-467.
- Vesonder, R. F., Gasdorf, H., and Peterson, R. E. 1993. Comparison of the cytotoxicities of *Fusarium* metabolites and *Alternaria* metabolite AAL toxin to cultured mammalian cell lines. *Arch. Environ. Contam. Toxicol.* 24:473-477.
- Walton, J. D. 1996. Host-selective toxins: Agents of compatibility. *Plant Cell* 8:1723-1733.
- Wang, H., Jones, C., Ciacci-Zanella, J. R., Holt, T., Gilchrist, D. G., and Dickman, M. B. 1996a. Fumonisin and *Alternaria alternata* *lycopersici* toxins: Sphinganine analog mycotoxins induce apoptosis in monkey kidney cells. *Proc. Natl. Acad. Sci. U.S.A.* 93:3461-3465.
- Wang, H., Li, J., Bostock, R. M., and Gilchrist, D. G. 1996b. Apoptosis: A functional paradigm for programmed plant cell death induced by a host-selective phytotoxin and invoked during development. *Plant Cell* 8:375-391.
- Wong, E. Y., Hironaka, C. M., and Fischhoff, D. A. 1992. *Arabidopsis thaliana* small subunit leader and transit peptide enhance the expression of *Bacillus thuringiensis* proteins in transgenic plants. *Plant Mol. Biol.* 20:81-93.
- Wu, W. I., McDonough, V. M., Nickels, J. T., Ko, J. S., Fischl, A. S., Vales, T. R., Merrill, A. H., and Carman, G. M. 1995. Regulation of lipid biosynthesis in *Saccharomyces cerevisiae* by fumonisin B₁. *J. Biol. Chem.* 270:13171-13178.