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The plant disease resistance gene *Asc-1* prevents disruption of sphingolipid metabolism during AAL-toxin-induced programmed cell death

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Summary

The necrotrophic fungus *Alternaria alternata* f.sp. *lycopersici* infects tomato plants of the genotype *asc/asc* by utilizing a host-selective toxin, AAL-toxin, that kills the host cells by inducing programmed cell death. *Asc-1* is homologous to genes found in most eukaryotes from yeast to humans, suggesting a conserved function. A yeast strain with deletions in the homologous genes *LAG1* and *LAC1* was functionally complemented by *Asc-1*, indicating that *Asc-1* functions in an analogous manner to the yeast homologues. Examination of the yeast sphingolipids, which are almost absent in the *lag1Δlac1Δ* mutant, showed that *Asc-1* was able to restore the synthesis of sphingolipids. We therefore examined the biosynthesis of sphingolipids in tomato by labeling leaf discs with L-[3-³H]serine. In the absence of AAL-toxin, there was no detectable difference in sphingolipid labeling between leaf discs from *Asc/Asc* or *asc/asc* leaves. In the presence of pathologically significant concentrations of AAL-toxin however, *asc/asc* leaf discs showed severely reduced labeling of sphingolipids and increased label in dihydrosphingosine (DHS) and 3-ketodihydrosphingosine (3-KDHS). Leaf discs from *Asc/Asc* leaves responded to AAL-toxin treatment by incorporating label into different sphingolipid species. The effects of AAL-toxin on *asc/asc* leaflets could be partially blocked by the simultaneous application of AAL-toxin and myriocin. Leaf discs simultaneously treated with AAL-toxin and myriocin showed no incorporation of label into sphingolipids or long-chain bases as expected. These results indicate that the presence of *Asc-1* is able to relieve an AAL-toxin-induced block on sphingolipid synthesis that would otherwise lead to programmed cell death.

Abbreviations: DHS, dihydrosphingosine; PHS, phytosphingosine; 3-KDHS, 3-ketodihydrosphingosine; TLC, thin-layer chromatography.

Keywords: AAL-toxin, *Alternaria alternata* f.sp. *lycopersici*, ceramide, programmed cell death, sphinganine *N*-acyltransferase, sphingolipids.

Introduction

Programmed cell death is recognized not only as an important process in the natural development of plants (Greenberg, 1996), but also as an essential component of the plant defense response, often by a rapid type of cell death known as the hypersensitive response (Greenberg, 1997; Mittler, 1998). Such plant-pathogen interactions are commonly investigated within the confines of the gene-for-gene model in which pathogen and host specificity are tightly associated. A group of fungi however, the host-selective toxin producing fungi, do not fit within this model. In these

species, a major factor in pathogenicity is the production of a toxin that in most cases is capable of inducing cell death in susceptible plants (reviewed by Markham and Hille, 2001; Walton, 1996).

Alternaria alternata f.sp. *lycopersici* is one such host-selective toxin-producing fungus that is able to infect tomato plants of the *asc/asc* genotype (Grogan *et al.*, 1975). A compatible interaction, in this case, is mediated through the production of AAL-toxin (Gilchrist and Grogan, 1976). Insensitivity to the toxin and resistance to the fungus segregate

together through many generations, and as a result it has been assumed that insensitivity to AAL-toxin and resistance to the fungus are conferred by a single gene at the locus designated *Asc* (Clouse and Gilchrist, 1987). It is known that application of AAL-toxin to sensitive leaves is sufficient to cause death in leaves, and that cell death occurs, in this instance, by programmed cell death (Wang *et al.*, 1996; Witsenboer *et al.*, 1988). The unclear factor is what initiates the cell death program in susceptible leaves and the way resistant plants remain insensitive to the effects of the toxin.

Evidence from plant and mammalian studies using AAL-toxin and the structurally analogous toxin fumonisin B1, has shown that the toxin inhibits sphinganine *N*-acyltransferase (acyl-CoA-dependent ceramide synthase) (Lynch, 1999; Wang *et al.*, 1991), the central enzyme of ceramide biosynthesis (Figure 1). The ceramide synthesis pathway in yeast and animals is known to be a powerful generator of a variety of signals involved in maintaining cellular homeostasis (Hannun *et al.*, 2001). Ceramide, sphingolipid-associated long-chain bases and their phosphorylated derivatives are signalling molecules associated with apoptosis or cellular proliferation in animals (Hannun and Luberto, 2000; Hannun *et al.*, 2001) and cell growth inhibition (Kim *et al.*, 2000) and heat shock in yeast (Dickson *et al.*, 1997; Jenkins *et al.*, 1997; Wells *et al.*, 1998).

Consistent with this, tomato leaf discs treated with AAL-toxin or fumonisin B1 have been shown to accumulate the long-chain bases dihydrosphingosine (DHS) and phytosphingosine (PHS) (Abbas *et al.*, 1994). Leaf discs from plants of genotype *Asc/Asc* also showed increases in long-chain bases, although to a lesser extent than leaf discs from sensitive tissues. This difference in the generation of long-chain bases makes them a likely candidate for the generation of programmed cell death signals in plants. However, reports of *in vitro* measurements of the sphinganine *N*-acyltransferase reaction from *Asc/Asc* and *asc/asc* plants have suggested that the enzymes from both plants are equally sensitive to inhibition by AAL-toxin (Gilchrist *et al.*, 1994). Thus, the origin of the signal for programmed cell death remains unclear.

A step towards understanding the role of *Asc* in preventing AAL-toxin-induced programmed cell death is the recent cloning of *Asc-1* (Brandwagt *et al.*, 2000). *Asc-1* confers resistance to AAL-toxin in hairy root cultures of *asc/asc* tomato (Brandwagt *et al.*, 2000) and expression of *Asc-1* in *Nicotiana umbratica*, a plant that is also susceptible to *A. alternata* f.sp. *lycopersici* infection (Brandwagt *et al.*, 2001), indicates that *Asc-1* is also able to confer resistance to infection by the fungus (Brandwagt *et al.*, 2002). However, to date no evidence has been produced to show what

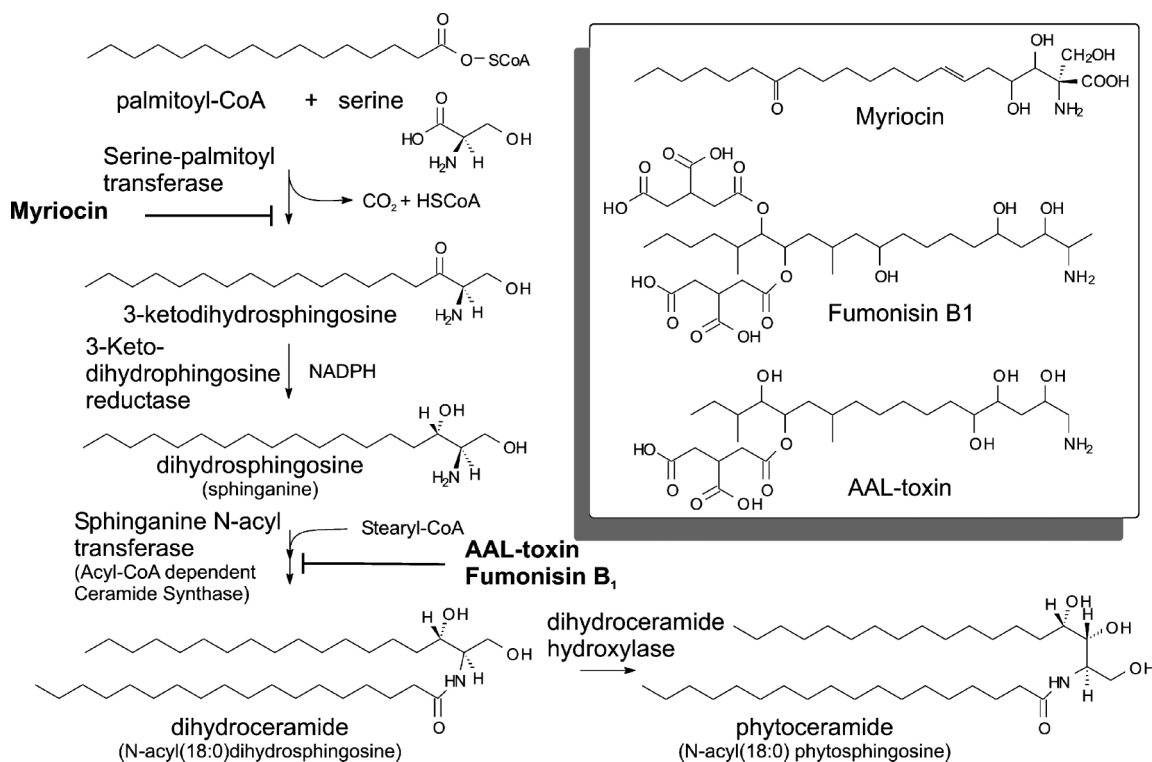


Figure 1. Enzymatic steps involved in long chain base and ceramide biosynthesis in fungi (Dickson and Lester, 1999). All the enzyme activities are known to occur in higher plants (Lynch, 1999; Sperling *et al.*, 2001). The toxins AAL-toxin and fumonisin B1 are inhibitors of sphinganine *N*-acyltransferase whereas myriocin prevents the formation of long-chain bases by inhibiting serine palmitoyl transferase.

Asc-1 does to confer insensitivity to AAL-toxin and resistance to *A. alternata* f.sp. *lycopersici*.

Asc-1 is homologous to a group of genes found in a wide variety of eukaryotes from yeast to humans (Brandwagt *et al.*, 2000; Jiang *et al.*, 1998). Characteristic of these genes are the multiple transmembrane domains and a highly conserved motif designated as the Lag1p motif (Jiang *et al.*, 1998). Deletion of both *LAG1* and *LAC1*, the only two homologues in *Saccharomyces cerevisiae*, leads to a slow growth or lethal phenotype depending upon the genetic background of the yeast (Barz and Walter, 1999; Jiang *et al.*, 1998). The slow growth phenotype was previously associated with cell wall defects and delayed transport of glycosylphosphatidylinositol (GPI)-anchored proteins (Barz and Walter, 1999). Recent studies in *S. cerevisiae* have revealed that Lag1p and Lac1p are essential for the sphinganine *N*-acyltransferase reaction in yeast (Guillas *et al.*, 2001; Schorling *et al.*, 2001). This coupled with the fact that AAL-toxin specifically inhibits sphinganine *N*-acyltransferase and it seems highly probable that *Asc-1* will be important for plant sphingolipid biosynthesis. To date, only circumstantial evidence exists for a role of sphingolipids in plant programmed cell death.

The data in this report show that *Asc-1* is partially able to complement the *lag1Δlac1Δ* phenotype in yeast and this is associated with the ability of *Asc-1* to restore sphingolipid biosynthesis. By using the same strategy of feeding L-[3-³H]serine that is used in yeast, we were able to label tomato sphingolipids and long-chain bases. Applying this strategy to transgenic *asc/asc* tomato plants containing the *Asc-1* gene, we show that *Asc-1* confers insensitivity to AAL-toxin and resistance to *A. alternata* f.sp. *lycopersici*, probably due to its role in sphingolipid metabolism in plants.

Results

Asc-1 partially complements the lag1Δlac1Δ double deletion in yeast

Asc-1p is 19% identical to *LAG1p* and 44% similar over the whole protein (19% identity, 39% similarity to *LAC1p*). It is,

however, somewhat shorter, 308 amino acids compared to 411 (*LAC1p* is 418 amino acids). As a result, prediction of transmembrane domains shows one less transmembrane domain. The transmembrane profiles of these proteins have been suggested as a conserved feature but it is not known if it is important for function (Jiang *et al.*, 1998). Despite this, the most conserved domain, the *LAG1p* motif, is predicted to span an inside to outside transmembrane domain in both *Asc-1p* and the yeast proteins, suggesting that the function may be conserved.

In order to test whether *Asc-1* is able to complement the deletion of the homologous genes, *LAG1* and *LAC1*, in yeast, the *Asc-1* cDNA (accession number AJ312131) was introduced under the control of the *GAL1* promoter into the diploid, double heterozygous, *lag1Δlac1Δ* strain WBY600 (Table 1). Transformation of the diploid strain was necessary owing to the extremely low transformation efficiency of the haploid *lag1Δlac1Δ* WBY616 strain (Barz and Walter, 1999). After sporulation, haploids with ADE⁺ HIS⁺ URA⁺ phenotypes were selected creating yeast strains pMVHAsc (Table 1). A second strain that harbors the *Asc-1* cDNA, WBYAscKK was also created, but with a modified 3'-end to engineer an ER retention signal (KXKXX). Unlike the yeast genes, this signal is not present in tomato *Asc-1* and it was introduced into the plant cDNA in order to increase the ER retention efficiency in yeast. The absence of the *LAG1* and *LAC1* alleles and the presence of the *lag1Δ::HIS3* and *lac1Δ::ADE2* insertions in the ADE⁺ HIS⁺ URA⁺ haploids were confirmed by genomic polymerase chain reaction (PCR) analysis (data not shown).

The growth rates of the *lag1Δlac1Δ* haploid double deletion mutant WBY616, the wild-type strain W303-1A and our complementing strains WBYAsc and WBYAscKK were compared (Figure 2a,b). The *lag1Δlac1Δ* mutant WBY616 grew slowly on minimal media when glucose was provided as a carbon source, but unexpectedly, was unable to grow using galactose as a carbon source. Our strains WBYAsc and WBYAscKK were able to grow using galactose as a carbon source, as was the wild-type strain W303-1A. This was because galactose induced the expression of *Asc-1* (data not shown), which complemented, albeit partially, the growth defects of the *lag1Δlac1Δ* yeast double deletion mutant and its inability to utilize galactose as a carbon

Table 1 Yeast strains used in this study

Strain	Genotype	Reference/source
W303-1A	MAT α <i>ade2-1 his3-11 leu2-3,-112 trp1-1 ura3-1 can1-100</i>	Barz and Walter (1999)
WBY 600	Diploid <i>ade2-1 his3-11 leu2-3,-112 trp1-1 ura3-1 can1-100</i> heterozygous for <i>lag1Δ:: HIS3</i> and <i>lac1Δ:: ADE2</i>	Barz and Walter (1999)
WBY 616	MAT α <i>ade2-1 his3-11 leu2-3,-112 trp1-1 ura3-1 can1-100 lag1Δ:: HIS3 lac1Δ:: ADE2</i>	Barz and Walter (1999)
WBYHis	<i>ade2-1 his3-11 leu2-3,-112 trp1-1 ura3-1 can1-100 lag1Δ:: HIS3 lac1Δ:: ADE2</i> (pMVHis)	This study
WBYAsc	<i>ade2-1 his3-11 leu2-3,-112 trp1-1 ura3-1 can1-100 lag1Δ:: HIS3 lac1Δ:: ADE2</i> (pMVHAsc)	This study
WBYAscKK	<i>ade2-1 his3-11 leu2-3,-112 trp1-1 ura3-1 can1-100 lag1Δ:: HIS3 lac1Δ:: ADE2</i> (pMVHAscKK)	This study

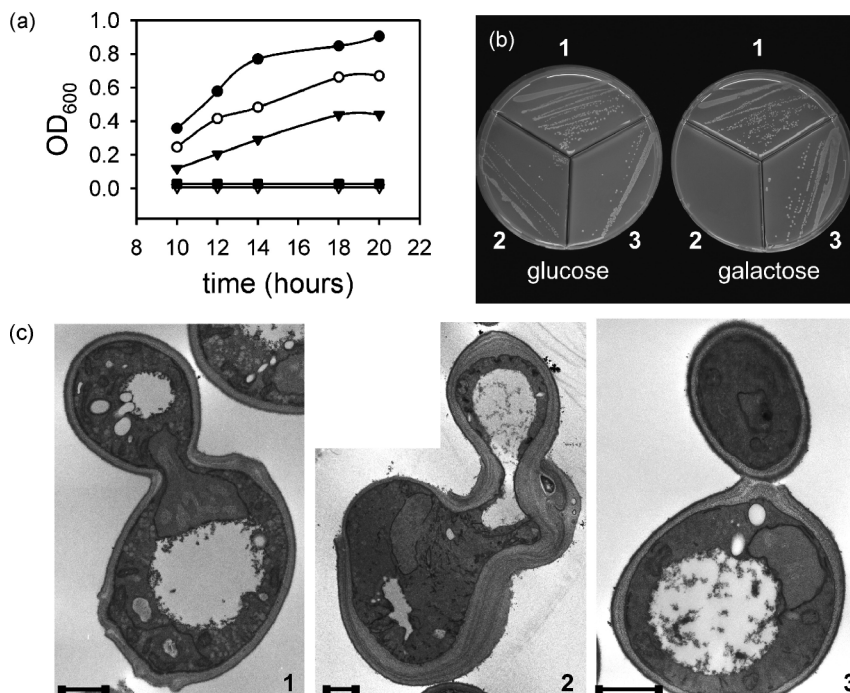


Figure 2. Complementation of the growth and cell wall defects in *lag1Δlac1Δ* yeast strain WBY616 by *Asc-1*.

(a) Growth curves of yeast strains: W303-1A, wild type (●); WBY616, *lag1Δlac1Δ* (▽); WBYHis, *lag1Δlac1Δ* pMVHis (■); WBYAsc, *lag1Δlac1Δ* gal1::*Asc-1* (▼); WBYAscKK, *lag1Δlac1Δ* gal1::*AscKK* (○), at 29 °C in minimal media containing the appropriate supplements and 2% galactose. The curves are representative of three independent experiments.

(b) Plates show the growth of yeast strains: (1) W303-1A; (2) WBY616; and (3) WBYAscKK after 48 h on minimal media containing the appropriate supplements and either 2% glucose or galactose as the sole carbon source. The growth defects of the *lag1Δlac1Δ* double-deletion mutant WBY616 are so severe that when galactose is the sole carbon source, no growth is observable. When utilizing glucose WBY616 grows slower than the wild-type W303-1A or our complementing strain WBYAscKK.

(c) Electronmicrographs showing that expression of *Asc-1* restores the severe cell wall phenotype of *lag1Δlac1Δ* yeast: (1) WBY600 (diploid, double heterozygous, *lag1Δlac1Δ* strain) showing wild-type morphology (bar 1 μm); (2) WBY616 (haploid *lag1Δlac1Δ* mutant) showing severe wall thickening and layering (bar 1 μm); and (3) WBYAscKK (haploid, *lag1Δlac1Δ* gal1::*AscKK* complementing strain) displaying a normal cell wall structure (bar 1 μm).

source. The complementing strain WBYAscKK, carrying the *Asc-1* gene modified by a dilysine motif at the C-terminus, grew on average 30% faster than the strain without, suggesting that ER localization enhances the ability of this protein to complement the lack of yeast LAG1p or LAC1p.

The low growth rate of the *lag1Δlac1Δ* mutant WBY616 strain is also associated with cell wall defects (Barz and Walter, 1999). We examined the complementing strain WBYAscKK by electron microscopy and compared it with the *lag1Δlac1Δ* haploid mutant WBY616 and the diploid heterozygous *lag1Δlac1Δ* WBY600 strains. Normal cell walls consist of an electron translucent layer with a thin, electron-dense inner and outer layer (1 in Figure 2c). The double deletion strain shows characteristic thickening of the translucent layer interspersed with electron-dense layers, suggestive of multiple wall layers, one upon the other (2 in Figure 2c). The cell wall defects present in the *lag1Δlac1Δ* mutant WBY616 were absent in our complementing strain (3 in Figure 2c). This supports the growth rate data and confirms that *Asc-1* can complement the deletions in *LAG1* and *LAC1*.

Asc-1 is involved in sphingolipid metabolism

Asc-1 confers insensitivity in tomato to AAL-toxin and fumonisin B1, toxins that inhibit the enzyme sphinganine *N*-acyltransferase. Deletion of the yeast *Asc-1* homologues *LAG1* and *LAC1* leads to delays in GPI-anchored protein transport, a process known to be dependent upon ceramide biosynthesis (Barz and Walter, 1999; Hovarth *et al.*, 1994). Therefore, we examined the incorporation of L-[3-³H]serine into sphingolipids in three individual transformants of our complementing strain, WBYAscKK, and compared it with the *lag1Δlac1Δ* mutant WBY616 and wild-type W303-1A strains.

When compared to the wild-type yeast strain W303-1A, *lag1Δlac1Δ* mutant WBY616 showed reduced incorporation of label into sphingolipids (Figure 3, lanes 3 and 4). Also faintly detectable was the presence of labeled long-chain bases 3-ketodihydrosphingosine (3-KDHS), DHS and PHS, which were not detected in the wild-type strain W303-1A. Crucially, the complementing strain WBYAscKK shows restoration of sphingolipid biosynthesis and no detectable, labeled precursors. Additionally a novel, probably

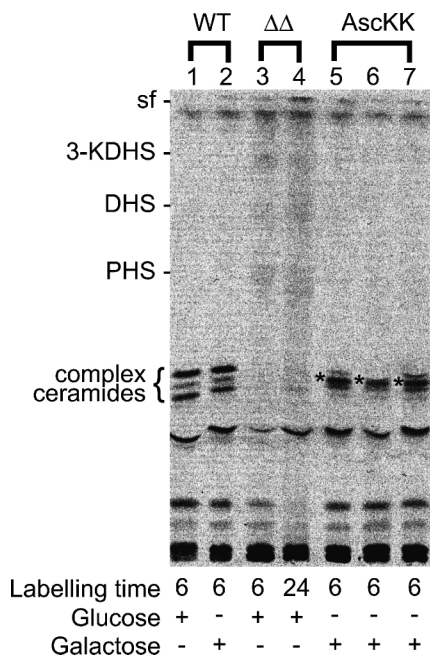


Figure 3. Effect of complementation by *Asc-1* on the incorporation of label into yeast sphingolipids.

Yeast sphingolipids were labeled by growing yeast in SD media + 2% glucose or galactose with L-[3-³H]serine and separated by TLC. For strains W303-1A (WT) and WBYAscKK (*AscKK*) the starting culture density was OD₆₀₀ of 0.1 and the labeling time 6 h. Under these conditions, strain WBY616 ($\Delta\Delta$) grows much more slowly. To compensate for this, the culture was either started at a higher density (OD₆₀₀ of 0.3) and grown for 6 h (lane 3) or started at the same density (OD₆₀₀ of 0.1) and grown until it reached the same final density, 24 h (lane 4). The position of the novel lipid synthesized by the complementing strain WBYAscKK is marked with an asterisk. The picture is representative of three independent experiments.

sphingolipid, species is synthesized in the complementing strain (asterisk, Figure 3, lanes 5–7). These results indicate that *Asc-1p* is functionally homologous to *Lag1p* and *Lac1p* and is able to restore sphingolipid biosynthesis in the *lag1Δlac1Δ* mutant WBY616 strain.

Analysis of resistance in transgenic *Asc-1* plants

The physiological and biochemical evidence from our yeast studies suggests that sphingolipid metabolism is at the heart of the mechanism of resistance conferred by *Asc-1* to *A. alternata* f.sp. *lycopersici*. In order to understand this further, susceptible tomato plants of genotype *asc/asc* were transformed with a genomic copy of the *Asc-1* gene. Southern blot analysis showed that the resulting transgenic plants contained single and multiple copies of the transgene (Figure 4). Digestion of tomato DNA with *HindIII* fragments the *Asc* and *asc* alleles into two bands, one of 2.4 kb and another of 4.2 or 3.9 kb depending on the genotype, the *asc* allele being the smaller of the two (Figure 4, lanes 1 and 16, Brandwagt *et al.*, 2000). The Southern analysis in Figure 4, therefore confirms that all plants were of genotype

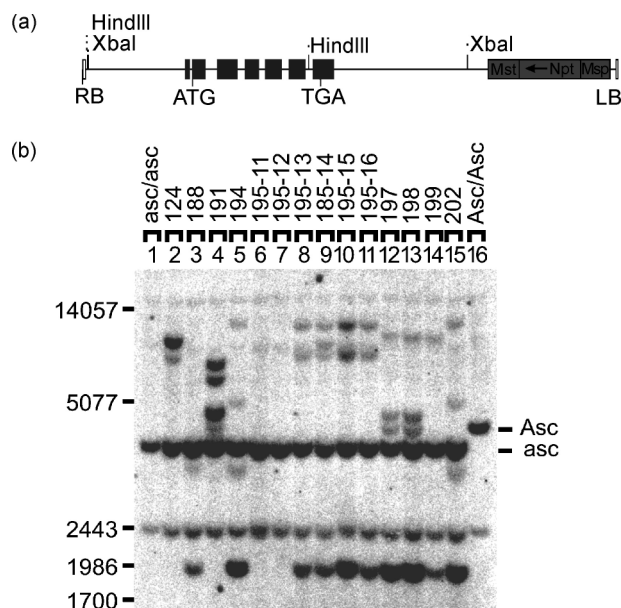


Figure 4. Southern analysis of transgenic *asc/asc* plants containing the *Asc-1* transgene.

(a) T-DNA construct for transformation of *Asc-1*. The *XbaI* genomic fragment includes the promoter, exons (black boxes) and terminator for *Asc-1*. The ATG start and TGA stop are marked along with the position of the *XbaI* and *HindIII* sites. *npt*, neomycin phosphotransferase gene; *MspI*, mannopine synthase 5' region; *MstI*, mannopine synthase 3' region; LB, left border; RB, right border.

(b) Genomic DNA digested with *HindIII* shows an RFLP between *asc/asc* (lane 1) and *Asc/Asc* (lane 16) genotypes. All transgenics are originally *asc/asc*. Most transgenics show a 1.9-kb *HindIII* band from digest between the *Asc-1* transgene and the RB of the T-DNA. The reason why transgenics 124 and 191 do not show a 1.9-kb band is unknown, however, these clearly contain the *Asc-1* transgene.

asc/asc before transformation. The 1.9-kb band in the Southern analysis of the transgenics is a fragment derived from a *HindIII* site in the transgene and in the right border of the T-DNA, the left border fragment is of variable size depending on the next *HindIII* site in the genomic DNA. When challenged, none of the transgenic plants ($n=18$, nine independent lanes, Figure 4, lanes 2–5, 12–15) showed signs of infection by *A. alternata* f.sp. *lycopersici* as judged by the lack of disease symptoms (data not shown). This was identical to *Asc/Asc* plants ($n=6$), but in contrast to *asc/asc* plants ($n=6$) which all showed stem cankers and leaf necrosis. The transformation process itself did not lead to resistance, as other transgenic plants of the *asc/asc* genotype remained susceptible to infection. Progeny of the transgenic plants were also examined (Figure 4, lanes 6–11) and toxin-sensitive plants were identified. Only the toxin-sensitive progeny were susceptible to infection by *A. alternata* f.sp. *lycopersici*. Southern analysis shows that the transgenes in these susceptible progeny had segregated out (Figure 4, lanes 6 and 7). This demonstrates that susceptibility of tomato to *A. alternata* f.sp. *lycopersici* is conferred solely by the absence of a functional *Asc-1* gene.

Sphingolipid metabolism in resistant and susceptible plants

To investigate the role of the *Asc-1* gene in sphingolipid biosynthesis in plants, we incubated leaf discs derived from young leaflets of *Asc/Asc* or *asc/asc* genotype tomato plants with L-[3-³H]serine. Total sphingolipids and long-chain bases were extracted and analyzed by thin-layer chromatography (TLC) in a manner analogous to yeast (Figure 5a, lanes 1 and 2). Under these conditions, *Asc/Asc* and *asc/asc* leaf discs did not show significant differences ($P > 0.3$) in the incorporation of label into 3-KDHS or DHS (Figure 5b). This could be explained by functional redundancy, tomato contains, like yeast, multiple homologues

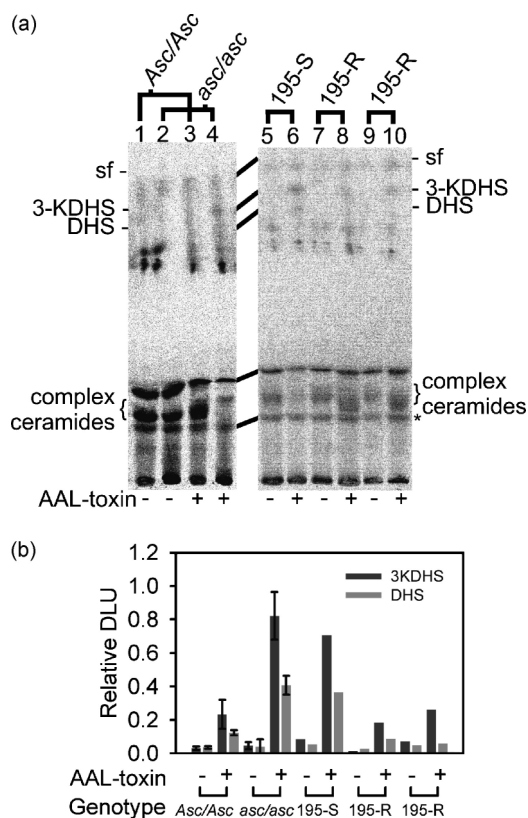


Figure 5. Effect of AAL-toxin on the incorporation of label into sphingolipids in leaf discs from *Asc/Asc* and *asc/asc* tomato plants.

(a) TLC of lipids extracted from leaf discs of genotype *Asc/Asc* (lanes 1 and 3) or genotype *asc/asc* (lanes 2 and 4) after incubating for 18 h in the presence of L-[3-³H]serine with (lanes 3 and 4) or without (lanes 1 and 2) AAL-toxin. Lanes 5-10 shows the response of progeny from the transgenic line 195. Sensitive progeny (195-S) respond like *asc/asc* plants whereas insensitive progeny (195-R) behave like *Asc/Asc* plants. The presence of sphingolipids at the marked position on the TLC plate was confirmed by mass spectrometry (see text for details). Other compounds were identified by their R_f values (3-KDHS) and their position in relation to authentic standards (DHS). (b) The relative levels of 3-KDHS and DHS were determined by digital radiography with respect to the lipid marked with an asterisk. Quantification was from four independent samples, bars show \pm standard deviation. Also shown is the quantification of the transgenic feeding experiments in Figure 5(a) lanes 5-10.

of *Asc-1*, at least one of which is expressed in the leaves (EMBL accession number AJ416474).

The difference in sphingolipid metabolism between *Asc/Asc* and *asc/asc* plants was further investigated by incubating leaf discs from *Asc/Asc* and *asc/asc* plants with AAL-toxin. Upon treatment with pathologically significant levels of AAL-toxin (200 nm), *Asc/Asc* leaf discs responded with the incorporation of label into extra sphingolipids (Figure 5a, lane 3). Conversely, *asc/asc* leaf discs showed drastically reduced incorporation of label into sphingolipids (Figure 5a, lane 4). In both cases, significantly ($P < 0.01$) elevated levels of compounds that run towards the top of the TLC, including DHS and 3-KDHS, were visible, although they were significantly ($P < 0.002$) more pronounced in the extracts from *asc/asc* leaf discs (Figure 5b). The region from the TLC plate labeled sphingolipids in Figure 5 was analyzed by tandem mass spectrometry (data not shown). A precursor ion scan for the N'' ion (dC18:0 mass 266) detected the presence of the Z_0 ion for ceramide with C18, C20 and C24 acyl chains (mass 567, 596, 652, detected at m/z 567, 597 and 653, respectively) (Sullards *et al.*, 2000; Sullards, 1999). A similar scan for the N'' ion (dC18:2 mass 262) also found the corresponding Z_0 ion for C:20 and C:24 acyl chains (mass 592 and 648, detected at m/z 593 and 649, respectively). The M ions corresponding to phosphoinositol dC18:2, C:24 and (glycosyl)₂-dC18:2, C24 were also detected (mass 834 and 972., detected at m/z 835 and 972, respectively). Compounds towards the bottom of the TLC are the hydrophilic head groups from non-sphingolipids, the acyl groups having been removed by methanolysis, such as phosphatidylserine and other glycerolipids. Incorporation of label into these compounds was unaffected by incubation with AAL-toxin during these experiments. This difference in response between *Asc/Asc* and *asc/asc* genotypes shows that insensitivity to AAL-toxin and probably resistance to *A. alternate* f.sp. *lycopersici*, correlates with the ability to continue incorporating long-chain bases into sphingolipids despite the presence of AAL-toxin.

This was further demonstrated by examining the incorporation of L-[3-³H]serine into sphingolipids from leaf discs of the transgenic tomato plants in the presence and absence of AAL-toxin. Progeny of line 195 were identified that were sensitive to the toxin due to segregation of the transgenes (e.g. Figure 4, lanes 6 and 7). Incorporation of L-[3-³H]serine into sphingolipids in leaf discs from such plants was inhibited by AAL-toxin in a similar fashion to *asc/asc* plants (Figure 5a, lanes 5 and 6, Figure 5b). Insensitive progeny of line 195 showed incorporation of L-[3-³H]serine into sphingolipids despite the presence of AAL-toxin (Figure 5a, lanes 7-10, Figure 5b). Five other transgenic lines were also checked for their ability to incorporate L-[3-³H]serine into sphingolipids in the presence of AAL-toxin and in all cases the results resembled *Asc/Asc*

plants (data not shown). These data indicate that *Asc-1* conferred resistance to *A. alternata* f.sp. *lycopersici* is closely linked to its effects on sphingolipid metabolism in the presence of AAL-toxin.

Sphingolipid metabolism and cell death in plants

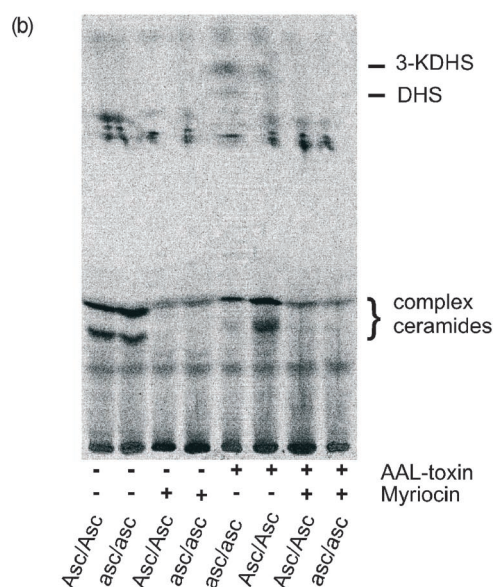
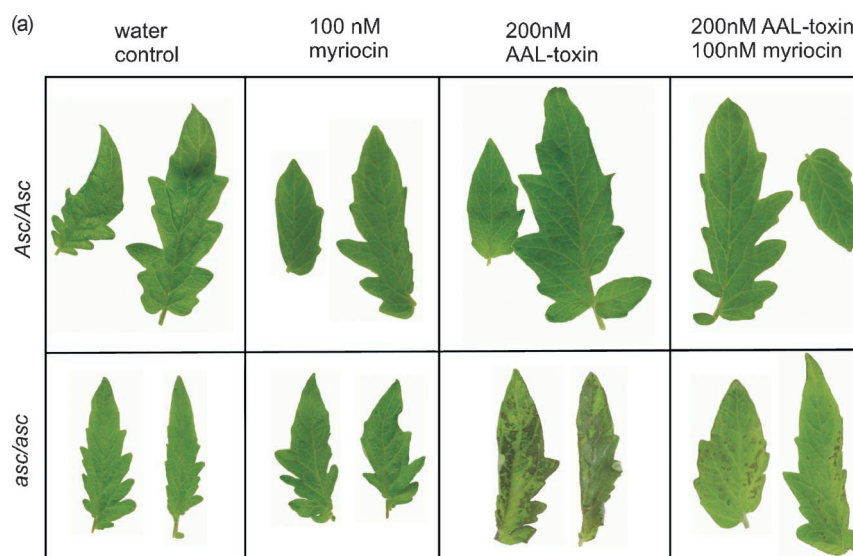
The previous experiments do not distinguish whether the increases in DHS and 3-KDHS are important for AAL-toxin-induced programmed cell death, or if the decrease in sphingolipids is also important. To investigate the importance of these two classes of compounds in programmed cell death we simultaneously treated *asc/asc* leaflets with AAL-toxin and myriocin. Myriocin is a potent inhibitor of serine palmitoyltransferase, the first enzyme of sphingolipid biosynthesis (Figure 1), but is not by itself capable of

inducing cell death in *asc/asc* tomato leaflets for the test period (48–72 h) and concentrations (0.1–10 μM) (Figure 6a). Therefore, a decrease in *de novo* ceramide synthesis is not, by itself, capable of inducing programmed cell death. Treatment with myriocin and AAL-toxin together substantially prevented cell death in *asc/asc* tomato leaves compared to AAL-toxin treatment alone (Figure 6a). As expected, the increased incorporation of label into DHS and 3-KDHS observed with AAL-toxin treatment alone, was inhibited (Figure 6b). The levels of incorporation into DHS and 3-KDHS observed with AAL-toxin- and myriocin-treated *asc/asc* leaf discs were identical to untreated plants, whereas leaf discs treated with AAL-toxin alone showed elevated levels as seen previously. This difference correlates with the protection from AAL-toxin-induced programmed cell death in the leaflet bioassay and suggests that the increase

Figure 6. Effects of simultaneous treatment of *Asc/Asc* and *asc/asc* tomato leaflets with myriocin and AAL-toxin.

(a) Leaflets were incubated on filter papers saturated with tap water containing AAL-toxin and/or myriocin for 48 h at 24°C under 16 h light/8 h dark regime. Under these conditions, no necrosis was visible as a result of myriocin treatment in the *asc/asc* or *Asc/Asc* background. AAL-toxin induced severe necrosis in *asc/asc* leaflets as expected, but none in *Asc/Asc*. Simultaneous treatment with myriocin and AAL-toxin substantially reduced the amount of necrosis in *asc/asc* leaves.

(b) Tomato leaf discs from *asc/asc* plants were incubated with L-[3-³H]serine with or without AAL-toxin and/or myriocin. Lipids were extracted and processed as before. The incorporation of label into sphingolipid and long chain base species is blocked in the presence of myriocin. The picture is representative of two independent experiments.



in these compounds could be important for programmed cell death.

Discussion

The yeast genes *LAC1* and *LAG1* have recently been shown to be essential for sphinganine *N*-acyltransferase activity in yeast (Guillas *et al.*, 2001; Schorling *et al.*, 2001). This paper looks at a homologous plant gene, *Asc-1*, identified for a very different reason, for its role in plant pathology as a disease resistance gene, and shows that *Asc-1* plays a similar if not identical role in sphingolipid biosynthesis in plants.

Deletion of the yeast *Asc-1* homologues *LAG1* and *LAC1* leads to a large reduction in sphingolipid biosynthesis (Figure 3) (Guillas *et al.*, 2001; Schorling *et al.*, 2001). These deletions are usually lethal except in the presence of a suppressor that is able to synthesize unusual, non-sphingolipid species (Guillas *et al.*, 2001). Residual ceramides are still synthesized due to the presence of the *YPC1* and *YDC1* genes (Schorling *et al.*, 2001). *YPC1* and *YDC1* encode ceramidases capable of synthesizing ceramide in an acyl-CoA-independent reaction, essentially a reversal of the hydrolysis reaction (Mao *et al.*, 2000).

Expression of the *Asc-1* gene in WBY616 leads to a substantial recovery in growth rate and abolishes the cell wall defects seen in this strain. Surprisingly, we found that the WBY616 strain is unable to grow when the only carbon source is galactose. The reason for this is not known, but it is also interesting to note that *asc/asc* plants are modified with respect to their sucrose uptake (Moussatos *et al.*, 1993).

Examination of the sphingolipids in these strains clearly shows that WBY616 does not incorporate wild-type amounts of label into sphingolipids, although incorporation of label into glycerolipids was unaffected. The expression of *Asc-1* clearly reversed this mutation demonstrating the functional homology of *Asc-1* with *LAG1/LAC1*. It is important to note that *Asc-1* also gave yeast the capability to synthesize a novel lipid species (asterisk, Figure 3). This is most likely a sphingolipid species judging by its position on the TLC. In this case, the difference between the novel lipid and other sphingolipids ought to be in the lipid backbone. This indicates that *Asc-1* may affect the synthesis of different ceramides than *LAG1* or *LAC1*.

The profile of incorporation of label into sphingolipids in leaf discs from *Asc/Asc* and *asc/asc* tomato plants supports the conclusion from the yeast data that *Asc-1p* is involved in ceramide metabolism. The fact that *Asc/Asc* and *asc/asc* sphingolipid patterns did not differ without AAL-toxin challenge is most likely due to the presence of two more *Asc* homologues in tomato (data not shown), at least one of which is expressed in tomato leaves. This is supported by the phenotype of a single *LAG1* or *LAC1* deletion in yeast which, although normally indistinguishable from wild type,

also has a conditional phenotype – hypersensitivity to calcofluor (TRIPLES database V7A12, Kumar *et al.*, 2000). Interestingly, the two *Asc* genotypes both responded to the AAL-toxin insult by altering the profile of incorporation of label into sphingolipids. Although *Asc/Asc* leaflets are phenotypically insensitive to 200 nM AAL-toxin they responded to this concentration with incorporation of label into different complex ceramides. Conversely, sensitive *asc/asc* leaf discs respond with decreased incorporation of label into complex ceramides and an increase in DHS and 3-KDHS (Figure 5).

Ceramide metabolism and signalling are recognized as important pathways in animals where they play multiple roles in growth regulation, stress signalling, apoptosis, membrane biology and protein sorting (Hannun *et al.*, 2001; Merrill *et al.*, 1996). Both ceramide and long-chain bases can operate as second messengers and trigger programmed cell death in animal systems (Kroesen *et al.*, 2001; Tolleson *et al.*, 1999; Yu *et al.*, 2001). In contrast, virtually nothing is known about the role of ceramide and long chain base signalling in plants (Gilchrist, 1997).

AAL-toxin induces programmed cell death in *asc/asc* tissues, so it could be assumed that a decrease in incorporation of label into sphingolipids is a result of cell death and not a cause. The arguments against the results being an effect of programmed cell death are: (i) incorporation of label into non-sphingolipids is not affected; (ii) at 200 nM AAL-toxin no cell death is phenotypically observable until 72 h after toxin exposure (Witsenboer *et al.*, 1988), we performed extractions at 18 h; (iii) up to 24 h after toxin exposure, no electrolyte leakage is observed indicating cells remain intact up to this point (Abbas *et al.*, 1994). There is one curious point however, previous observation of the long-chain bases in AAL-toxin-treated tomato tissues have reported increases in PHS and DHS (Abbas *et al.*, 1994). In these experiments, we observed the largest increase in 3-KDHS. The increase in this non-reduced, non-hydroxylated form could be explained by two scenarios (A) that the pathway of sphingolipid biosynthesis has become blocked also at the 3-ketodihydrospingosine reductase step or (B) that there is a decrease in available reducing power in the cell such that 3-KDHS accumulates. The feeding experiments were performed such that the tissue always had adequate light, oxygen and an extracellular supply of carbon. Additionally, accumulation of 3-KDHS only occurs in toxin challenged tissues, therefore eliminating scenario B. The reason for the difference between our observation and those previously reported (Abbas *et al.*, 1994) might lie in the methodology used. Our experiments show only the incorporation of label into new lipids and do not determine the absolute concentration of DHS or PHS. Indeed, in our experiments PHS was not observed. It has been reported that PHS is not a substrate for plant sphinganine *N*-acyltransferase (Lynch, 1999)

suggesting rather that it is a breakdown product from hydrolysis of ceramide. It is therefore possible that ceramide biosynthesis is blocked leading to a small rise in *de novo* synthesized DHS and a larger increase in 3-KDHS, and that ceramide hydrolysis also occurs leading to increases in, in our experiments unlabelled hence unobserved, DHS and PHS.

To understand if a general inhibition of *de novo* sphingolipid biosynthesis is enough to cause programmed cell death, we inhibited the first enzyme of *de novo* ceramide biosynthesis, serine-palmitoyltransferase, with myriocin. Previous studies with LLC-PK1 cells show that myriocin can reverse the fumonisin B1 increase of free long-chain bases (Schroeder *et al.*, 1994). Myriocin did not cause cell death, for the time of exposure and at the concentrations tested in the experiments here, but blocked, as expected, *de novo* sphingolipid biosynthesis. Myriocin was also partially able to rescue the cell death phenotype of AAL-toxin-treated *asc/asc* leaflets. This strongly indicates that long-chain bases or a derivative could be a cell death signal in AAL-toxin-induced programmed cell death (Merrill *et al.*, 1996). Incubation with myriocin cannot block the accumulation of long-chain bases resulting from hydrolysis of ceramide and if this occurs, it may explain the reduced amount of programmed cell death observed in this instance. Previous work has demonstrated that incubation with ceramide also rescues *asc/asc* plants from AAL-toxin-induced programmed cell death (Brandwagt *et al.*, 2000). In this case, external ceramide could compensate for the lack of inhibition of ceramide biosynthesis but not prevent the accumulation of long-chain bases from inhibition of sphinganine *N*-acyltransferase. Taken together, these results indicate an interaction between the levels of ceramide and long-chain bases with no single factor controlling the decision to enter programmed cell death.

An interesting question arises from these observations, what is the actual interaction between the AAL-toxin, a structural homologue of sphingoid long-chain bases, and Asc-1p from tomato? It is known that *asc-1* is a recessive allele and carries a two-base pair deletion in exon 2 that leads to a premature stop codon and presumably a non-functional protein (Brandwagt *et al.*, 2000). In our experiments *asc-1* was not able to complement *lag1Δlac1Δ* yeast double deletion mutant (data not shown). This evidence leads to the conclusion that in *asc/asc* plants there is no functional *asc-1p*, thus excluding direct AAL-toxin/*asc-1p* interaction in the *asc/asc* genetic background. A lack of Asc-1 protein therefore, makes *asc/asc* plants vulnerable to AAL-toxin whilst the presence of Asc-1p makes *Asc/Asc* plants insensitive. If we assume that the direct target of AAL-toxin is sphinganine *N*-acyltransferase, and the evidence presented here suggests that, then it would suggest that Asc-1p forms a component of or is associated with sphinganine *N*-acyltransferase and prevents inhibition by AAL-toxin.

The changes in sphingolipid profile in *lag1Δlac1Δ* yeast expressing the *Asc-1* gene suggest that the Asc-1p enables the sphingolipid biosynthesis pathway to utilize different substrates. The alteration in sphingolipid labeling seen in *Asc/Asc* plants also suggests a change in substrate utilization. The pattern observed could be explained by a form of sphinganine *N*-acyltransferase that does not utilize Asc-1p but perhaps one of its plant homologues. This form should be more susceptible to inhibition by AAL-toxin than a second form that has a different substrate preference and contains Asc-1p. In *asc/asc* plants therefore, the form of sphinganine *N*-acyltransferase that utilizes Asc-1p is not present and sphinganine *N*-acyltransferase is inhibited at low concentrations of AAL-toxin. In *Asc/Asc* plants then, the first form of sphinganine *N*-acyltransferase is inhibited at low AAL-toxin concentrations, but the form utilizing Asc-1p is not, thus, sphingolipid synthesis continues, although with a modified substrate profile. This model would be at odds with previous reports that sphinganine *N*-acyltransferase is equally sensitive to inhibition by AAL-toxin (Gilchrist *et al.*, 1994), but possibly the substrates used therein only measured one form of the enzyme. Without further detailed information about the assay used to obtain this data, it is not possible to understand exactly which reaction was being inhibited. Sphinganine *N*-acyltransferase can utilize a variety of substrates and our data indicate that *Asc-1* may be able to alter substrate incorporation into sphingolipids. Careful *in vitro* and *in vivo* dissection of the ceramide biosynthesis pathway will be required to understand the biochemical processes involved.

Accordingly, Asc-1p alone brings resistance to *A. alternata* f.sp. *lycopersici*. Transgenic *asc/asc* plants containing just a single copy of the *Asc-1* gene are fully resistant to *A. alternata* f.sp. *lycopersici* just like *Asc/asc* plants (van der Biezen *et al.*, 1994). Therefore, a single *Asc-1* gene is enough to continue sphingolipid biosynthesis in the presence of toxin secreted from the fungus. As is the case for other host-selective toxin producing fungi, this mechanism of resistance to *A. alternata* f.sp. *lycopersici* seems to be unique (Markham and Hille, 2001). The incorporation of label into sphingolipids in the *Asc-1* transgenic plants showed *Asc/Asc* profiles supporting the idea that resistance to *A. alternata* f.sp. *lycopersici* is via ceramide biosynthesis and susceptibility most likely is a result of a signal or signals derived from disruption of the ceramide biosynthesis pathway.

To our knowledge, this study presents the first *in vivo* measurements of sphingolipid biosynthesis in higher plants and demonstrates conclusively that AAL-toxin inhibits sphingolipids biosynthesis in leaf discs. The link between ceramide metabolism and programmed cell death in plants is a tantalizing one that needs further investigation. The evidence presented herein provides a basis for a future analysis of long chain base and ceramide biosynthesis in plants.

Experimental procedures

Isolation of *Asc-1* cDNA

mRNA was purified from tomato (*Lycopersicon esculentum* Mill, genotype *Asc/Asc* (Clouse and Gilchrist, 1987)) leaf total RNA using Oligotex (Qiagen Ltd., Crawley, West Sussex, UK). cDNA was synthesized (Superscript Choice System, Invitrogen BV, Breda) using an oligo dT primer, cloned into *EcoRI* pre-digested, dephosphorylated lambda ZAPII (Stratagene, Amsterdam), and packaged. Lambda phage containing the *Asc-1* cDNA were identified by screening plated phage with a ^{32}P -labeled RT-PCR product generated from tomato leaf cDNA using sense primer 5'-ATGAAAAACCTGGATCACATCG-3' and antisense primer 5'-GTGCTGCCTCTACAAGAAATAC-3'. The primers were based on the known genomic sequence. DNA was sequenced using the AFLExpress II (Amersham Biosciences, Roosendaal) or by GATC Biotech AG, Konstanz, Germany.

Complementation of *lag1Δlac1* yeast

The strains used in this study are listed in Table 1. The genetic background of all the strains was W303-1A. Standard yeast protocols and media were used throughout (Sherman, 1991). The *Asc-1*-coding region was amplified with sense primer 5'-AGATCTAAAAACCTGGATCACATCGCTG-3' and antisense primer 5'-GTGCTGCCTACAAGAAATAC-3'. The resulting PCR product was subcloned into pBluescript (Stratagene) and subsequently cloned into pMVHis as *BglII/XhoI* fragment to create the plasmid pMVHAsc. pMVHAsc is a yeast expression vector containing the *GAL1* promoter sequence, an *N*-terminal His-tag and URA selection (kindly provided by Martijn van Hemert, Institute of Molecular Plant Sciences, University of Leiden, the Netherlands). An alternative antisense primer 5'-TCATTACACCTGTCTTTGTGGTCATCATC-3' was used to modify the 3'-end to produce a KKKXX ER retention sequence. This was cloned in the same manner into pMVHis to create the plasmid pMVHAscKK. The haploid strains WBYAsc, WBYAscKK and WBYHis were produced by transformation of the diploid strain WBY600 with the plasmids pMVHAsc, pMVHAscKK or pMVHis, respectively. Haploid strains were generated by sporulation on minimal sporulation media, digestion for 1 h at 37°C with 2000 U lyticase (Sigma-Aldrich Chemie B.V., Zwijndrecht) followed by incubation with 20% ethanol for 40 min. Haploids with the *lag1Δ::HIS3* and *lac1Δ::ADE2* genotype and pMVHis URA⁺ vectors were selected on synthetic minimal (SD) media.

Electron microscopy

Cells were fixed in 1.5% KMnO₄ (w/v) in water for 20 min at RT, stained in 1% (w/v) uranylacetate in water for several hours and subsequently dehydrated in a graded series of ethanol and finally embedded in Epon. After ultra-thin sectioning the micrographs were taken on a Philips CM10 transmission electron microscope.

Labeling and extraction of yeast sphingolipids

Sphingolipids were labeled with L-[3- ^3H]serine (Amersham Biosciences) essentially as previously described (Oh *et al.*, 1997). Briefly, 3 ml cultures at an OD₆₀₀ of 0.1 were incubated in SD media with 50 μCi of L-[3- ^3H]serine for 6 h at 29°C. Lipids were extracted twice into 1 ml of ethanol:water:diethyl ether:pyridine:NH₄OH

(15:15:5:1:0.018) (Hanson and Lester, 1980). Lipids were dried under N₂ and subjected to mild alkaline methanolysis using 0.5 ml of monomethylamine reagent (70% of 33% methylamine solution (Sigma-Aldrich B.V.) with 30% water (Clarke and Dawson, 1981). The methanolysed lipids were dried under N₂ and dissolved in 0.1 ml of chloroform:methanol:H₂O (16:16:5).

Labeling and extraction of plant sphingolipids

Twenty leaf disks, 4 mm in diameter, were pre-incubated for 3 h in 5 ml of MS10 media, 0.05% 2-[N-morpholino] ethane sulphonic acid pH 5.6 (MES) with or without toxin(s). After pre-incubation, 25 μCi of L-[3- ^3H]serine was added and the leaf discs were incubated in the light, at room temperature with slow shaking (90 rpm), for 18 h. The extraction of plant sphingolipids was done as described by (Hanson and Lester, 1980) with modifications. The leaf discs were washed twice with MS10 media and ground under liquid N₂. The ground powder was mixed with 0.84 ml of 5% TCA and incubated on ice for 30 min. Concentrated ammonia was used to adjust the pH to 9.5 and 1.16 ml of ethanol:diethylether:pyridine (15:5:1 v/v) was added. Methanolysis was performed as described above for the yeast sphingolipids.

Thin layer chromatography

The lipid extracts were separated by TLC on a 20-cm silica gel 60F₂₅₄ plate (Merck Nederland B.V., Amsterdam) using chloroform:methanol:4.2N NH₄OH (9:7:2) as solvent phase. The sphingolipid standards were detected after spraying the TLC plate with 10% CuSO₄·5H₂O in 8% phosphoric acid and charring for 10 min at 160°C (Fewster *et al.*, 1969). The sphingolipid standards ceramide, DHS and PHS were from Sigma.

Autoradiography and quantification

Radioactivity was detected by exposure to an appropriate storage phosphor screen and detection via a Cyclone imager (Packard-Bioscience B.V., Groningen, the Netherlands). TLCs were digitally quantified by profile analysis and peak integration after background subtraction (OptiQuant Image Analysis Software, Packard-Bioscience B.V.). The results were tested for significant differences by using Student's *t*-test analysis for small samples, assuming a two-tailed distribution and paired data.

Transgenic tomato plants

A 3.9-kb *XbaI* gDNA fragment encoding *Asc-1* with 688 bp of 5' intergenic region and 1549 bp of 3' intergenic region was cloned into the binary vector pCGN1548 (McBride and Summerfelt, 1990). Tomato, genotype *asc/asc*, was transformed using *Agrobacterium tumefaciens* strain EHA105 as previously described (van Roekel *et al.*, 1993).

Fungal infections and toxin bioassays

A. alternata f.sp. *lycopersici* was maintained on V8 media (20% V8 (N.V. Campbel Foods Belgium S.A., Puurs, Belgium), 0.3% CaCO₃ solidified with 1.6% agar. Tomato plants were infected together in a small, contained, greenhouse as previously described (Grogan *et al.*, 1975).

AAL-toxin and myriocin were purchased from Sigma. The AAL-toxin stock solution, 100 μM, was prepared in water. The myriocin

stock solution, 2.5 mM, was prepared in 96% ethanol and 0.1% Tween-80. Leaflets were assayed for sensitivity to AAL-toxin and/or myriocin by placing the excised leaflet on a filter paper with 3 ml of water, 0.1% Tween-80 and the appropriate amount of AAL-toxin or myriocin in a sealed Petri dish as described previously (Clouse and Gilchrist, 1987).

Southern blot analysis

Genomic DNA was isolated from tomato plants using a CTAB protocol modified so that the extraction buffer contained 0.1% β -mercaptoethanol (Dean *et al.*, 1992). Southern blotting and hybridization were performed as described (Sambrook *et al.*, 1989). The blot was probed with an *Asc-1* cDNA labeled with [α - 32 P]dCTP by random priming. The blot was washed twice with $2 \times$ SSC 0.5% SDS at room temperature for 5 min, and with $0.2 \times$ SSC, 0.1% SDS at 68°C for 30 min.

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