Chapter 3

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

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In *The Plant Journal* (2002) 32, 561-572

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Summary

The nectrotrophic fungus A*lternaria* a*lternata* f.sp. l*ycopersici* 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. We examined the biosynthesis of sphingolipids in tomato by labelling leaf discs with L-[3-^{3}H]serine. In the absence of AAL-toxin, there was no detectable difference in sphingolipid labelling 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 labelling of sphingolipids and increased label in dihydrosphingosine and 3-ketodihydrosphingosine. 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.

Introduction

Programmed cell death is recognised not only as an important process in the natural development of plants (Greenberg, 1996), but also as an essential component of the plant defence 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 B_1, has shown that the toxin inhibits sphinganine *N*-acyltransferase (acyl-CoA dependent ceramide synthase) (Lynch, 2000; Wang *et al.*, 1991), the central enzyme of ceramide biosynthesis. 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 *et al.*, 2001; Hannun and Luberto, 2000) 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 B_1 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.*, 1995). 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 *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). 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.

By using feeding with L-[3-^{3}H]serine, 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.

Experimental procedures

Labelling and extraction of plant sphingolipids

Twenty leaf disks, 4mm in diameter, were pre-incubated for 3h in 5ml of MS10 media, 0.05% 2-[*N*-morpholino] ethane sulphonic acid (MES) pH 5.6 with or without toxin(s). After pre-incubation, 25µCi of L-[3-^{3}H]serine was added and the leaf discs were incubated in the light, at room temperature with slow shaking (90rpm), for 18 hours. 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 N_2. The ground powder was mixed with 0.84ml of 5% TCA and incubated on ice for 30min. Concentrated ammonia was used to adjust the pH to 9.5 and 1.16ml of ethanol:diethyether:pyridine (15:5:1 v/v) was added. Methanolysis was performed as described in Chapter 2 for the yeast sphingolipids.

Thin-layer chromatography (TLC)

The lipid extracts were separated by TLC on a 20cm silica gel 60_{F254} plate (Merck Nederland B.V., Amsterdam) using choroform:methanol:4.2N NH_4OH (9:7:2) as solvent phase. The sphingolipid standards were detected after spraying the TLC plate with 10% $CuSO_4x5H_2O$ 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 (Sigma-Aldrich B.V, St. Louis MI, U.S.A.).

Autoradiography and quantitation

Radioactivity was detected by exposure to an appropriate storage phosphor screen and detection via a Cyclone imager (PackardBioscience B.V., Groningen, The Netherlands). TLCs were digitally quantitated by profile analysis and peak integration after background subtraction (OptiQuant Image Analysis Software, PackardBioscience 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 3.9kb *Xba*I gDNA fragment encoding *Asc-1* with 688bp of 5' intergenic region and 1549bp 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% $CaCO_3$) 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 (Sigma-Aldrich B.V, St. Louis MI, U.S.A.). The AAL-toxin stock solution, 100µM, was prepared in water. The myriocin stock solution, 2.5mM, 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 3ml 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 labelled with [α-^{32}P]dCTP by random priming. The blot was washed twice with 2×SSC 0.5% SDS at room temperature for 5 minutes, and with 0.2×SSC, 0.1% SDS at 68°C for 30 min.

Results

Analysis of resistance in transgenic Asc-1 plants

The physiological and biochemical evidence from our yeast studies (chapter 2) 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 *as*c/*as*c 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 1). Digestion of tomato DNA with *Hin*dIII fragments the *Asc* and *asc* alleles into two bands, one of 2.4kb and another of 4.2kb or 3.9kb depending on the genotype, the *asc* allele being the smaller of the two (Figure 1, lanes 1 and 16, (Brandwagt *et al.*, 2000). The Southern analysis in Figure 1, therefore confirms that all plants were of genotype *asc*/*asc* before transformation. The 1.9kb band in the Southern analysis of the transgenics is a fragment derived from a *Hin*dIII 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 *Hin*dIII site in the genomic DNA. When challenged, none of the transgenic plants (n=18, nine independent lines, Figure 1 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 *As*c/*Asc* plants (n=6), but in contrast to *as*c/*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 *as*c/*asc* genotype remained susceptible to infection. Progeny of the transgenic plants were also examined (Figure 1, lanes 6-11) and toxin sensitive plants were identified. Only

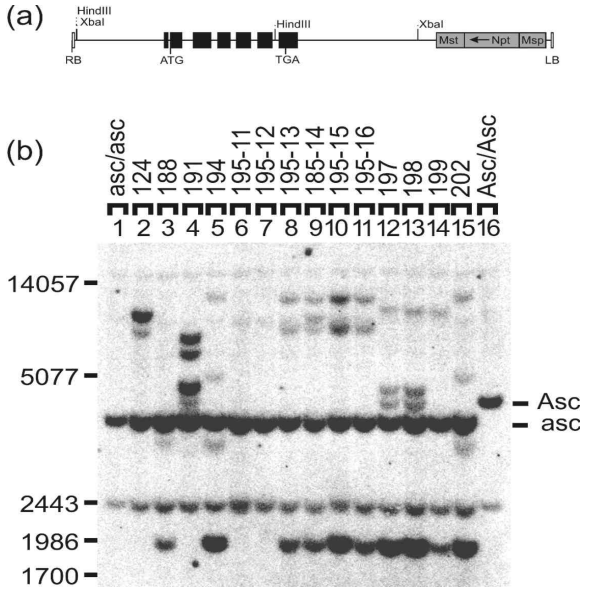

Figure 1. *Southern analysis of transgenic* asc/asc *plants containing the* Asc-1 *transgene.*
a) T-DNA construct for transformation of *Asc-1*. The *Xba*1 genomic fragment includes the promoter, exons (black boxes) and terminator for *Asc-1*. The ATG start and TGA stop are marked along with the position of the *Xba*1 and *Hin*dIII sites. Npt, neomycin phosphotransferase gene; Msp, Mannopine synthase 5' region; Mst, Mannopine synthase 3' region; LB, left border; RB, right border.
b) Genomic DNA digested with *Hin*dIII shows an RFLP between *asc/asc* (lane 1) and *Asc/Asc* (lane 16) genotypes. All transgenics are originally *asc/asc*. Most transgenics show a 1.9kb *Hin*dIII 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 1.9kb band is unknown, however these clearly contain the *Asc-1* transgene.

the toxin sensitive progeny were susceptible to infection by *A. alternata* f.sp. *lycopersic*i. Southern analysis shows that the transgenes in these susceptible progeny had segregated out (Figure 1, 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-^{3}H]serine. Total sphingolipids and long-chain bases were extracted and analysed by TLC in a manner analogous to yeast (Figure 2a, 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 2b). This could be explained by functional redundancy, tomato contains, like yeast, multiple homologues of *Asc-1*, at least one of which is expressed in the leaves (EMBL accession No. 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 (200nM), *Asc*/*Asc* leaf discs responded with the incorporation of label into extra sphingolipids (Figure 2a, lane 3). Conversely, *asc*/*asc* leaf discs showed drastically reduced incorporation of label into sphingolipids (Figure 2a, 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 2b). The region from the TLC plate-labelled sphingolipids in Figure 2 was analyzed by tandem mass spectrometry (data not shown). 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 phosphotidylserine 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 *Alternaria alternate* f.sp. *lycopersici*, correlates with the ability to continue incorporating long-chain bases into sphingolipids despite the presence of AAL-toxin.

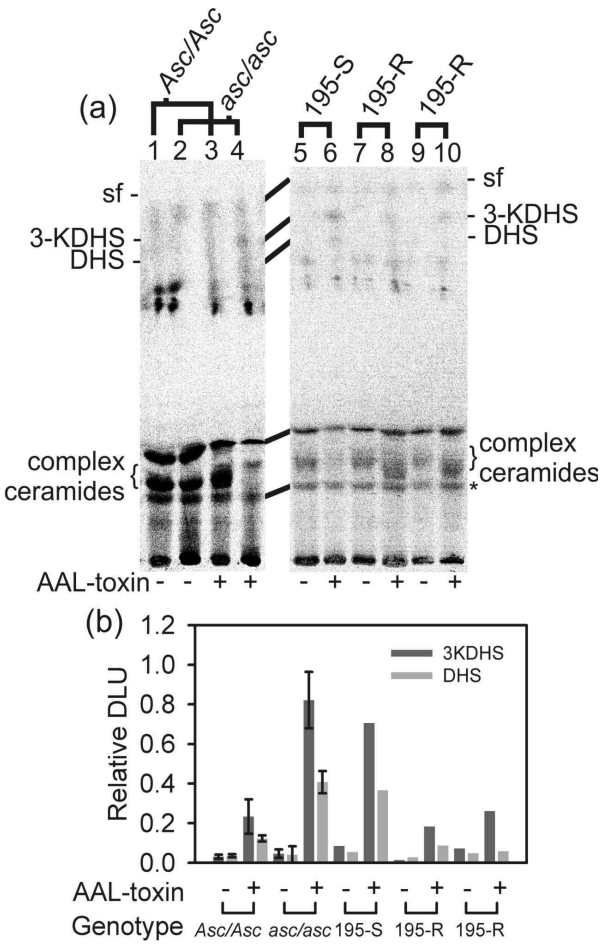

Figure 2. *Effect of AAL-toxin on the incorporation of label into sphingolipids in leaf discs from* Asc/Asc *and* asc/asc *tomato plants.*
(a) TLC of lipids extracted from leaf discs of genotype *Asc/Asc* (lanes 1 and 3) or genotype *asc/asc* (lanes 2 and 4) after incubating for 18h in the presence of L-[3-^{3}H]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 wherease 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. Quantitation was from 4 independent samples, bars show ± standard deviation. Also shown is the quantification of the transgenic feeding experiments in Figure 2a lanes 5-10.

This was further demonstrated by examining the incorporation of L-[3-^{3}H]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 1, lanes 6 and 7). Incorporation of L-[3-^{3}H]serine into sphingolipids in leaf discs from such plants was inhibited by AAL-toxin in a similar fashion to *asc/asc* plants (Figure 2a, lanes 5 and 6, Figure 2b). Insensitive progeny of line 195 showed incorporation of L-[3-^{3}H]serine into sphingolipids despite the presence of AAL-toxin (Figure 2a, lanes 7-10, Figure 2b). Five other transgenic lines were also checked for their ability to incorporate L-[3-^{3}H]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, but is not by itself capable of inducing cell death in asc/asc tomato leaflets for the tested times (48-72h) and concentrations (0.1 to 10μM) (Figure 3a). 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 3a). As expected, the increased incorporation of label into DHS and 3-KDHS observed with AAL-toxin treatment alone, was inhibited (Figure 3b). 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 tissue in the leaflet bioassay and suggests that the increase in these compounds could be important for programmed cell death.

Discussion

The yeast genes *LAG1* and *LAC1* 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.

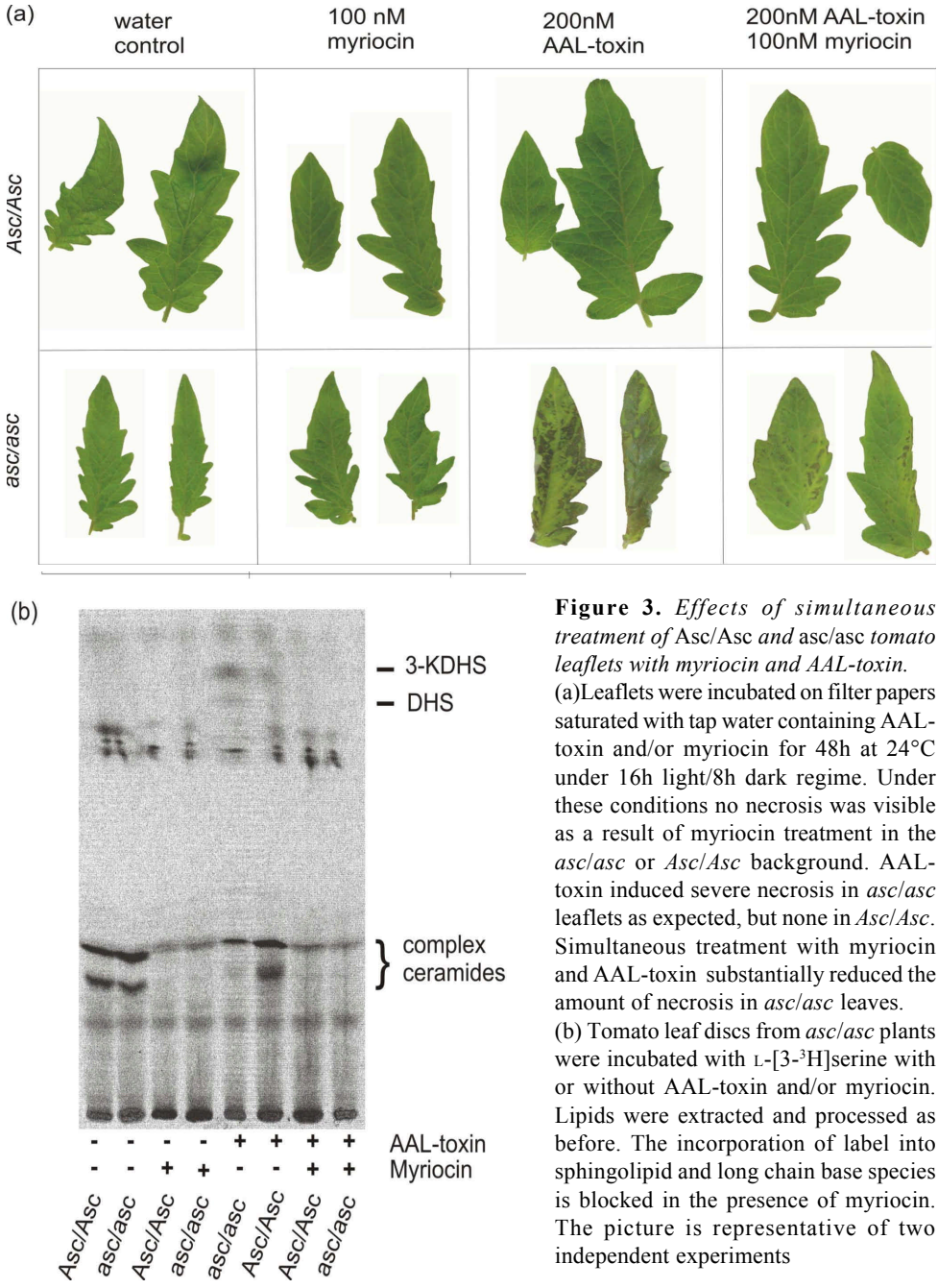

Figure 3. *Effects of simultaneous treatment of* Asc/Asc *and* asc/asc *tomato leaflets with myriocin and AAL-toxin.*
(a)Leaflets were incubated on filter papers saturated with tap water containing AAL-toxin and/or myriocin for 48h at 24°C under 16h light/8h dark regime. Under these conditions no necrosis 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-^{3}H]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

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 (chapter 2) 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 200nM AAL-toxin they responded to this concentration with incorporation of label into different complex sphingolipids. Conversely, sensitive *asc*/*asc* leaf discs respond with decreased incorporation of label into complex sphingolipids and an increase in DHS and 3-KDHS (Figure 2).

Ceramide metabolism and signalling are recognised 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 200nM AAL-toxin no cell death is phenotypically observable until 72 hours after toxin exposure (Witsenboer *et al.*, 1988), we performed extractions at 18 hrs; (iii) up to 24 hours 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-ketodihydrosphingosine 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 extra-cellular 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 than PHS is not a substrate for plant sphinganine *N*-acyltransferase (Lynch, 2000) 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* synthesised 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 B_1 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 *lag1D lac1D* 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 *lag1D lac1D* yeast expressing the *Asc-1* gene suggest that the Asc-1p enables the sphingolipid biosynthesis pathway to utilise different substrates. The alteration in sphingolipid labelling seen in *Asc*/*Asc* plants also suggests a change in substrate utilisation. The pattern observed could be explained by a form of sphinganine *N*-acyltransferase that does not utilise 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.*, 1995), 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 what 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 *Alternaria 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.

Acknowledgements

The authors thank Bert Venema for greenhouse assistance and Hjalmar Permentier for the mass spectrometry.

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