Functional Analysis of the Ceramide Synthase Gene ALT7, A Homolog of the Disease Resistance Gene Asc1, in the Plant Pathogen Alternaria alternata

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Abstract

The tomato pathotype of Alternaria alternata produces a host-specific AAL-toxin and causes Alternaria stem canker on susceptible tomato cultivars. AAL-toxin is a sphinganine-analog mycotoxin which induces apoptotic cell death in tomato cells and mammalian cells by inhibiting ceramide biosynthesis. Insensitivity to the AAL-toxin in resistant tomatoes and other plants is conferred by the Asc1 gene, a homolog of the yeast ceramide synthase gene Lag1. The ALT7 gene, a putative acyl-CoA-dependent ceramide synthase, was found to be located in the AAL-toxin biosynthetic (ALT) gene cluster of the tomato pathotype of A. alternata. ALT7 and Asc1 have the TLC (TRAM/Lag1/CLN8) domain characteristic of proteins involved in ceramide biosynthesis and are members of the LASS/Lag family. To test the hypothesis that ALT7 and Asc1, both of which are Lag1 ceramide synthase gene homologs, might share a common biological function as toxin tolerance genes, we have cloned and characterized ALT7. ALT7-deleted mutants were generated to investigate the effects on vegetative growth, sporulation, toxin-sensitivity, toxin-production and pathogenicity. The deletion of ALT7 has no deleterious effect on the toxin-producing pathogen, indicating that the gene does not act as a resistance/self-tolerance factor against the toxin in the biosynthetic gene cluster.

Keywords: Alternaria alternata; Tomato; AAL-toxin; Secondary metabolite; Gene cluster; Ceramide synthase

Introduction

Phytotoxins and mycotoxins produced by fungal plant pathogens are generally low molecular weight secondary metabolites that exert toxic effects on host plants and animals, respectively. Among the phytotoxins, host-specific toxins (HSTs) are critical determinants of pathogenicity or virulence in several plant–pathogen interactions [1,2]. The AAL-toxin and fumonisin are structurally related and were originally isolated from the tomato pathotype of Alternaria alternata (synonym A. alternata f. sp. lycopersici, synonym A. arborescens) and from Gibberella moniliformis, respectively [3,4,5,6].

AAL-toxin and fumonisin are sphinganine-analog mycotoxins (SAMs) that are harmful to some plant species and mammalian cells [7]. They cause apoptosis in susceptible tomato cells and mammalian cells by inhibiting ceramide biosynthesis [7,8,9]. In the interactions of the tomato plant with its pathotype of A. alternata, a major factor in pathogenicity is the production of host-specific AAL-toxin that is capable of inducing cell death only in susceptible cultivars [10,11,12]. In the tomato and other plants, insensitivity to AAL-toxin and fumonisin is conferred by the Asc1 (Alternaria stem canker resistance gene 1) gene, a homolog of the yeast longevity assurance gene Lag1, which mediates resistance to SAM-induced apoptosis by the production of an alternative ceramide [11].

Genes involved in the biosynthesis of secondary metabolites are typically clustered in filamentous fungi, some of which are plant pathogens [13,14,15,16]. The origin and evolution of these gene clusters, however, are largely unknown. The involvement of horizontal gene transfer (HGT) in the evolution of fungal secondary-metabolite gene clusters has been discussed [16]. The recent sequencing of the genomes of many fungi has revealed that genes involved in secondary metabolite biosynthesis are arranged in gene clusters [13,14]. This suggests that there are common architectures of gene clusters for the biosynthesis of fungal secondary metabolites. The typical cluster includes genes for enzymes such as polyketide synthases (PKS), transcription factors and metabolite resistance and/or self-tolerance genes [14,15]. This characteristic is useful for the identification of putative secondary metabolite gene clusters with the genomic data for a given fungus.

The AAL-toxin biosynthetic gene (ALT) cluster in the tomato pathotype of A. alternata was discovered by the draft sequencing of the genome of the tomato pathotype As-27 strain and subsequent comparison with the corresponding sequences of the fumonisin biosynthetic gene (FUM) cluster in G. moniliformis. The ALT cluster includes at least 13 genes, such as Type I PKS, cytochrome P450 monoxygenase, dehydrogenase and aminotransferase genes, all of which showed similarity to the genes in the FUM cluster [17,18,19,20]. Among these, we focused on ALT7, a putative acyl-CoA-dependent ceramide synthase gene. ALT7 shows similarity to FUM17 and FUM18 in the FUM cluster. Those genes are members of the mammalian LASS (longevity assurance homolog 1 of yeast Lag1) family and are homologous to the yeast Lag1 (longevity assurance gene 1) gene. Members of this family contain the TLC (TRAM/Lag1/CLN8) domain typical of proteins involved in ceramide synthesis and lipid regulation [8,21,22,23,24]. Interestingly, ALT7 also displayed similarity to the...

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AAL-toxin and disease resistance gene *Ascl* of the tomato and other plants [11]. Yeast *Lag1* and *Lac1* (longevity assurance gene cognate 1) are thought to encode ceramide synthase, the target enzyme of SAMs, and each gene can compensate for the absence of the other such that the deletion of either gene does not affect ceramide synthase activity; however, the deletion of both genes markedly impairs activity [21,25]. *Asc1* partially compensated for the growth defect in the *Lag1/Lac1* deleted yeast strains, indicating a common function of these plant and yeast ceramide synthase genes [8,22].

Taken together, these observations suggest the working hypothesis that *ALT7* in the *ALT* cluster of the tomato pathotype and the tomato toxin-resistance gene *Ascl*, both of which are *Lag1* ceramide synthase gene homologs, might share common biological and pathological functions. The toxin-producing plant pathogen and the host tomato plant share homologous genes: one for toxin self-tolerance and the other for toxin and/or disease resistance. To test this hypothesis, we have cloned and characterized the *ALT7* gene and generated *ALT7*-deleted mutants to investigate the effects of the deletion on vegetative growth, sporulation, toxin-sensitivity/tolerance, toxin-production and pathogenicity.

**Materials and Methods**

**Fungal strains and cultures**

The tomato pathotype of *Alternaria alternata* (synonym *A. alternata* Esp. *lycopersici*, synonym *A. arborescens*) As-27 strain was used in this study as the wild-type strain. The wild-type strain and the transformants derived from the pathotype were maintained on potato dextrose agar (PDA) (Difco) slants or in 20% glycerol as mycelial fragments at -80°C. The isolates were cultured on V8 juice agar medium for the production of conidia or in potato dextrose broth (PDB) for genomic DNA preparations. For analysis of AAL-toxin production, the strains were cultured on Richards’ medium.

**DNA and RNA isolation and cDNA synthesis**

For the extraction of DNA, fungi were grown in 50 mL of PDB in 100-mL Erlenmeyer flasks at 25°C for 2 days on a orbital shaker (120 r.p.m.). The resulting mycelia were ground in liquid nitrogen using a mortar and pestle. Total genomic DNA was extracted from the mycelia as described previously [10]. Total RNA for expression analysis was prepared from fungal mycelia grown under the same conditions described above. Total RNA was extracted using the RNaseasy Plant Mini Kit (Qiagen) following the manufacturer’s instructions. Total RNA was treated with DNase (Takara-Bio) to remove traces of contaminating DNA and 1 μg was converted into cDNA using the PrimeScript RT-PCR Kit (Takara-Bio) using random 6-mer primers according to the manufacturer’s instructions. Using the resulting cDNA, the primer pair *ALT7inF/*ALT7inR (Table 1) was used to amplify an internal sequence of *ALT7*.

**Isolation, gene targeting, and complementation of *ALT7***

The sequences of the PCR primers used in this study are shown in Table 1. The gene encoding the ceramide synthase gene *ALT7* (GenBank accession number AB666460) in the tomato pathotype *A. alternata* was determined by analyzing the draft sequence of the As-27 strain. The size of the full-length *ALT7* gene is 1593 bp. The scheme used for constructing the gene disruption vector and for generating the mutants is illustrated in Figure 2. PCR primer pairs *ALT7FA/*ALT7AR and *ALT7BF/*ALT7BR were used to amplify the flanking regions of the *ALT7* gene from the genomic DNA of the *A. alternata* As-27 strain. The p71sfi1 plasmid, which contains a hygromycin B phosphotransferase gene (*hph*) cassette, was used for amplification of the marker gene with the *fush*pH* and *fushpH* primers. The gene disruption construct was prepared by fusion PCR as described previously [26] with the outermost primer pair *ALT7FA/*ALT7BR, using a mixture of the PCR fragments, 5'-ALT7, 3'-ALT7 and the *hph* cassette. The PCR was performed using a Thermal Cycler Dice TP650 (Takara-Bio) or a MyCycler 170-9703JA (Bio-Rad Laboratories) thermal cycler with an initial denaturing step at 95°C, followed by 30 cycles of 15 s at 95°C, 15 s at 90°C, and 30 s at 72°C, and a final step of 5 min at 72°C. The final fused products

**Table 1: oligonucleotide primers used in this study.**

| Primers | Sequences (5'-3') |
|---------|------------------|
| ALT7AF | gagcccccttcacacatcc |
| ALT7AR | atccagctgctcagactcgtgctgggagcataag |
| ALT7BF | atcgagagctctagacagattgctgctgggcatctgctg |
| ALT7BR | tggctgttagatgagacc |
| HphF   | gagctgctgagagttgctt |
| HphR   | gatlgtgacattgcctgctg |
| ALT7FmF | gctatgtgctagtgacacc |
| ALT7mR | tacatccagcgaggaagagac |
| ALT7homoF | ggtgcccgctagccgttcgctgagtaagc |
| HphhomomR | caaatctgctgagtgagactagag |
| fushpH | gatcgcatgcatgctgctgctgctgctgagtaagc |
| fushpHR | aacctctgctgctgctgagtaagc |
| ALT7comF | caaatctgctgaggaagagac |
| ALT7comR | aacctctgctgaggaagagac |
were purified with the QIAquick Kit (Qiagen) before transformation into the A. alternata tomato pathotype As-27. For transforming the A. alternata strains, fungal protoplasts were prepared according to a previously described method [10] with modifications. Protoplasts (80 μl) were transformed with the disruption vectors by methods described previously [10,17].

Three different pairs of primers were used to identify the ALT7-deleted mutants from the hygromycin B-resistant colonies. First, a pair of primers for the hph cassette was used to verify the insertion of the vectors. Then, two pairs of the primers (ALT7homoF/hphhomoR and ALT7inF/ALT7inR) were used to interrogate the integration of the hph cassette by a double-crossover homologous recombination event at the ALT7 locus. Putative disruptants yielding the expected diagnostic amplification fragments (Figure 2) were purified by single-sparse isolation.

For the genetic complementation of ALT7, the ALT7 open reading frame with 5’- and 3’-franking sequences from ALT7 was amplified with the primers ALT7comF and ALT7comR using a high-fidelity DNA polymerase (Takara-Bio). The resulting fragment (2420 bp) was purified with the QIAquick Kit (Qiagen) and introduced into to ALT7-deleted strain T1 by co-transformation with the pl199 plasmid conferring resistance to geneticin [27]. Geneticin-resistant transformants were grown on a PDA-containing hygromycin B and geneticin at 50 μg/ml and 100 μg/ml, respectively. The expression of ALT7 in the transformant (T1C) was determined by RT-PCR as described above.

**Assays for pathogenicity, AAL-toxin production and vegetative growth**

Pathogenicity and toxin production of the wild-type and transformant strains of A. alternata were assessed as described previously [10,12]. The quantification of AAL-toxin Tp by HPLC was performed with pre-column derivatization of the toxin with o-phthalaldehyde as described previously [12,28]. To examine the colony growth and the morphology of the mutants, all strains were grown on PDA at 25°C for 4 days. agar blocks (3 mm in diameter) carrying mycelia were prepared from the resultant colonies and inoculated onto PDA. After incubation at 25°C for 4 days, colony growth and morphology were observed.

**Results and Discussion**

**Identification and targeted disruption of ALT7 in the tomato pathotype A. alternata**

The ALT7 gene (GenBank accession number AB66460), a putative acyl-CoA-dependent ceramide synthase in the tomato pathotype of A. alternata was discovered by analyzing the draft sequence of the As-27 strain. The full-length A. alternata ALT7 sequence is 1593 bp and encodes a protein of 432 amino acids. ALT7 showed 30% identity and 51% similarity (E-value = 8e^-16) with the yeast Lgl1 (longevity assurance gene 1)(AA215759) [21], and 26% identity and 45% similarity (E-value = 3e^-13) with the tomato Asc1 (Alternaria stem canker resistance gene 1) (AA675188) [11] at the deduced amino acid level. Phylogenetic analysis indicates that Alt7 belongs to the “Bar-type” ceramide synthase family (Figure 1A) [29,30]. Alt7 shows 44% identity and 61% similarity (E-value = 2e^-18) with the Aspergillus nidulans BarA (ANID_04332), and 42% identity and 62% similarity (E-value = 1e^-5) with the Fusarium graminearum Bar1 (FGSG_09423.3) [29,30]. Alt7 also possesses 31% identity and 47% similarity (E-value = 3e^-11) with Fum17 (AAN74820), and 40% identity and 60% similarity (E-value=5e^-06) with Fum18 (AAN74821) [20]. FUM17 and FUM18 are the Lag1 homolog genes found in the biosynthetic gene cluster of mycotoxin fumonisin B1, an inhibitor of ceramide synthase produced by the plant pathogenic fungus Gibberella manihotis [20].

Alt7 has the TLC (TRAM/Lag1/CLN8) domain characteristic of proteins involved in ceramide biosynthesis and lipid regulation, such as the mammalian LASS family, CLN8 and yeast Lag1/Lac1 [23,24]. A conserved Lag1p motif [33,31] shared only by LASS/Lag homologs is located in the AAL-toxin biosynthetic family (ALT7) [22,23]. The structural analysis of ALT7 revealed that this gene is a member of the LASS/Lag family of ceramide synthesis genes. The finding that ALT7 is located in the AAL-toxin biosynthetic gene cluster (ALT cluster) [17,19], together with the similarity of ALT7 with the AAL-toxin-resistant gene Ascl in tomato plants, indicated the possibility that ALT7 acts as a self-tolerance gene for AAL-toxin in the tomato pathotype. To characterize the function of ALT7 in the pathogen and to examine our hypothesis, a gene-targeting approach was employed to replace the ALT7 gene in the wild-type strain As-27 with the hph marker gene.

A targeting vector containing partial fragments of ALT7 was constructed for the disruption of the gene through homologous
were examined. In addition, an
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necrotic lesions within 3 days after inoculation. The number and size
disruption mutant and the

strain of

deletion strain T1 (Figure 3D). These results indicate that the deletion

mutant appeared subjectively and equally as “healthy” as the wild-type

ceramide synthesis in the susceptible tomato cultivar [8,11,12,23]. The

medium contains 50 µg/ml of AAL-toxin, which is over 1000 times

toxin by the pathogen was assessed by culturing the mutant strain on

ALT7 showed the same growth characteristics as the wild-type strain and the

mutants (Figures 3A,3B). The

showed the same growth characteristics as the wild-type strain (Figure 4B). The toxin production of these

strains was further examined by quantitative HPLC analysis (Figure 4C). The results indicated that the deletion of ALT7 does not affect the pathogen’s ability to produce the AAL-toxin. In G. moniliformis, disruption mutants of FUM17 and FUM18 also produced fumonisins at levels similar to the wild-type strains [20].

Taken together, these data indicate that the deletion of the ceramide synthase gene ALT7, which is the homolog of the tomato AAL-toxin-resistant gene Ascl and is located in the ALT cluster of the tomato pathotype of A. alternata, has no discernable deleterious effect on the toxin-producing pathogen. These results do not support our hypothesis that the toxin-producing pathogen and the disease-resistant plants share a common gene for toxin tolerance. The deletion of ALT7 in the tomato pathotype also has no effect on toxin production, even though the gene is located in the toxin biosynthetic gene cluster of the pathogen. Considering the role of each constitutive gene in secondary metabolite gene clusters, such as toxin gene clusters in fungi [13,14,15,16], ALT7 might have played an important role for self-protection against the toxin at the time when the gene cluster first originated in a pathogen. Later in evolution, duplication or mutation of the original gene might have caused genetic redundancy of the ALT7 gene. We have identified two additional homologous genes belonging to the LASS/Lag family in the pathogen’s genome by draft sequencing of the As-27 strains. Future work should focus on the specific function of these ceramide synthase

Phenotypic characterization of ALT7-targeted and –complemented strains

The effects of the ALT7 disruption on the vegetative growth of the AAL-toxin-producing A. alternata were examined. In addition, an ALT7-complementation strain (T1C) generated by the re-introduction of the ALT7 region into the mutant T1 was used for comparison. The results of colony growth and expression analysis are shown in Figure 3.

The effects of ALT7 deletion on conidiation and vegetative growth on an agar medium were observed. Agar blocks from colonies grown on PDA were transferred onto V-8 juice agar media and grown at 25°C for 14 days, and the plates were then placed under BLB lamps. There were no significant differences in the conidial yields (data not shown) and vegetative growth rates between the wild-type strain and the ALT7 mutants (Figures 3A,3B). The ALT7-complementation strain also showed the same growth characteristics as the wild-type strain and the ALT7 mutant (Figures 3A,3B). The self-protective ability against AAL-toxin by the pathogen was assessed by culturing the mutant strain on medium containing a high concentration of AAL-toxin. The high-toxin medium contains 50 µg/ml of AAL-toxin, which is over 1000 times higher than the concentration needed to induce necrosis and impair ceramide synthesis in the susceptible tomato cultivar [8,11,12,23]. The mutant appeared subjectively and equally as “healthy” as the wild-type strain, while the susceptible tomato leaves showed severe necrosis on the selective medium (Figure 3C). The expression of ALT7 in the wild-type As-27 and complementation strain T1C were confirmed by RT-PCR analysis (Figure 3D). ALT7 expression was not detected in the deletion strain T1 (Figure 3D). These results indicate that the deletion of ALT7 causes no detectable deficits in the vegetative and reproductive properties of the toxin-producing pathogen.

Pathogenicity and AAL-toxin production of ALT7-targeted strains

The pathogenicities of the wild-type and mutant strains were tested by inoculating conidia of each strain onto young detached leaves of the susceptible tomato cultivar Aichi first. The wild-type, the ALT7-disruption mutant and the ALT7-complementation strain all caused necrotic lesions within 3 days after inoculation. The number and size of the lesions were nearly identical on all of the leaves (Figure 4A). The strains were cultured on rice medium to assess their ability to produce AAL-toxin. After two to three weeks of growth, the toxin was extracted with 20 ml of 50 % acetonitrile. The extracts were filtered and stored at -20°C. The production of AAL-toxin by the culture was determined using a leaf necrosis bioassay with susceptible tomato plants. The results showed that the ALT7 mutant exhibited the same toxin productivity as the wild-type strain (Figure 4B). The toxin production of these strains was further examined by quantitative HPLC analysis (Figure 4C). The results indicated that the deletion of ALT7 does not affect the pathogen’s ability to produce the AAL-toxin. In G. moniliformis, disruption mutants of FUM17 and FUM18 also produced fumonisins at levels similar to the wild-type strains [20].

Taken together, these data indicate that the deletion of the ceramide synthase gene ALT7, which is the homolog of the tomato AAL-toxin-resistant gene Ascl and is located in the ALT cluster of the tomato pathotype of A. alternata, has no discernable deleterious effect on the toxin-producing pathogen. These results do not support our hypothesis that the toxin-producing pathogen and the disease-resistant plants share a common gene for toxin tolerance. The deletion of ALT7 in the tomato pathotype also has no effect on toxin production, even though the gene is located in the toxin biosynthetic gene cluster of the pathogen. Considering the role of each constitutive gene in secondary metabolite gene clusters, such as toxin gene clusters in fungi [13,14,15,16], ALT7 might have played an important role for self-protection against the toxin at the time when the gene cluster first originated in a pathogen. Later in evolution, duplication or mutation of the original gene might have caused genetic redundancy of the ALT7 gene. We have identified two additional homologous genes belonging to the LASS/Lag family in the pathogen’s genome by draft sequencing of the As-27 strains. Future work should focus on the specific function of these ceramide synthase
Figure 4: Pathogenicity and AAL-toxin production of the ALT77-disrupted strains. (A) Pathogenicity test by spore inoculation with A. alternata As-27 wild type (WT), ALT77-disrupted (T1) and ALT77-complemented (T1C) strains. The leaves were inoculated with a spore suspension (10^6 conidia/ml) and incubated in a moist chamber at 25°C for 3 days. (B) Leaf necrosis bioassay for AAL-toxin production by WT, T1 and T1C. The leaves of the susceptible tomato cultivar Aichi-first were treated with culture filtrates of each strain at 25°C for 3 days. (C) HPLC chromatograms of culture filtrates of WT, T1 and T1C. The strains were grown on rice for 15 days. Samples of each fungus were dried and HPLC chromatograms of culture filtrates of each strain at 25°C for 3 days. The OPA derivatization and HPLC analysis were described previously [12,28]. The peaks for AAL-toxin Tp are indicated by arrows.

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