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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 of the deletion 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 toxin 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 monooxygenase, 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 *Asc1* 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 strain, 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 *Asc1*, 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* f.sp. *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 an 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 RNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. Total RNA was treated with DNaseI (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 ALT7AF/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 *fushphF* and *fushphR* primers. The gene disruption construct was prepared by fusion PCR as described previously [26] with the outermost primer pair ALT7AF/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 of 5 min at 95°C, followed by 30 cycles of 15 s at 95°C, 15 s at 59°C, and 30 s at 72°C, and a final step of 5 min at 72°C. The final fused products

Primers	Sequences (5'- 3')
ALT7AF	gagccctttcacaacttcc
ALT7AR	atcaggtcgatgctagcatccttgtggacaccacatag
ALT7BF	atgcgagtgctaccagatgtgtttccagtacgcttgcg
ALT7BR	tgctgtcttagatgcagacc
HphF	gacgtctgtcgagaagtttc
HphR	gtattgaccgattccttgcg
ALT7inF	gtctatgtggtgtccacaag
ALT7inR	tcactcaccgcgaaaaggac
ALT7homoF	gtgggccacgcaggcctgtctgcgctaagcca
HphhomoR	caatagctttgggacgatgcaag
fushphF	gatgctagcatcgacctgatttacactttatgcttccg
fushphR	acatctggtagcactcgcatcttcgctattacgcca
ALT7comF	caacatgaaagtggacaccg
ALT7comR	acttgtctgctatggtacgc

Table 1: Oligonucleotide primers used in this study.

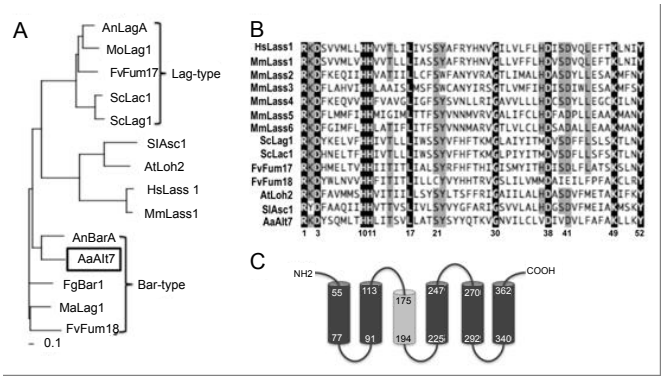

Figure 1: Phylogenetic analysis of *Lag1* homologs, the Lag1p motif and predicted membrane topology of *ALT7*. (A) Neighbor-joining phylogenetic analysis of the protein sequences of *Lag1* homologs. All *Lag1* homologs were aligned using ClustalW (Version 1.83). The phylogram was constructed using the neighbor-joining method with bootstrap support (1000 repetitions) and Poisson correction. All sequences are designated according to their annotation format or known protein name. (B) Amino acid alignment of the Lag1p motif. A highly conserved region of 52 amino acids in each protein is known as the Lag1p motif [23]. The black font indicates complete sequence identity, and the grey font indicates a sequence identity of at 70%. Accession numbers are exhibited in parentheses. *F. verticillioides* (FvFum17; AAN74820 and FvFum18; AAN74821), *A. nidulans* (AnLagA; AN2464 and AnBarA; AN4332), *Magnaporthe orizae* (MoLag1; XP_359588), *Metharizium acridium* (MaLag1; EFY90987), *S. cerevisiae* (ScLag1; AAA21579 and ScLac1; NP_012917), *Solanum lycopersicum* (SlAsc1; AAF67518), *Arabidopsis thaliana* (AtLoh2; NP_188557), human (HsLass1; AAD16892) and mouse (MmLass1-6; NP_619588, NP_084065, XP_620510, NP_080334, NP_082291 and Q8C172, respectively) sequences were obtained from NCBI. *F. graminearum* (FgBar1; XP_389599) sequences were obtained from the Fungal Genome Initiative (http://www.broad.mit.edu/annotation/fungi/fgi/). (C) A putative transmembrane domain. Transmembrane domains of the Alt7p were predicted using the SOSUI engine ver. 1.11 (http://bp.nuap.nagoya-u.ac.jp/sosui/sosui_submit.html). The black and grey cylinders indicate primary and secondary types of helices, respectively. The number in each cylinder refers to the corresponding amino acid sequence.

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-spore 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 pII99 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 T_A 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 = $8e^{-33}$) with the yeast *Lag1* (longevity assurance gene 1) (AAA21579) [21], and 26% identity and 45% similarity (E-value = $3e^{-13}$) with the tomato *Asc1* (Alternaria stem canker resistance gene 1) (AAF67518) [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 = $2e^{-89}$) with the *Aspergillus nidulans* BarA (ANID_04332), and 42% identity and 62% similarity (E-value = $1e^{-67}$) with the *Fusarium graminiarum* Bar1 (FGSG_09423.3) [29,30]. Alt7 also possesses 31% identity and 47% similarity (E-value = $3e^{-31}$) with Fum17 (AAN74820), and 40% identity and 60% similarity (E-value=$5e^{-66}$) 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 moniliformis* [20].

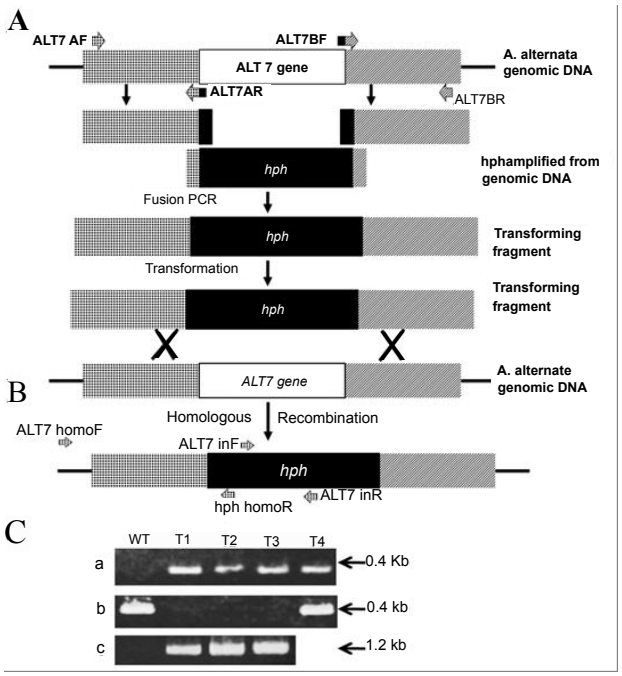

Figure 2: Deletion strategies for the *ALT7* in the tomato pathotype of *A. alternata*. (A) A fusion PCR method was used to construct the *ALT7* replacement vector. All PCR primers used in this figure are listed in Table 1. The 5' region of *ALT7* was amplified by PCR with the primer pair ALT7AF/ ALT7AR, and the 3' region was amplified with the primer pair ALT7BF/ ALT7BR; the sizes of the PCR products were 540 and 600 bp, respectively. The *hph* gene was amplified with the primer pair fushphF/fushphR. The three PCR products were then used as a template for fusion PCR using the primer pair ALT7AF/ALT7BR, and the resulting PCR product was used for transformation. (B) The *ALT7* locus before and after replacement of *ALT7* with the marker gene *hph*. (C) PCR analysis of the gene replacement events in *ALT7* in the wild-type strain As-27 (WT) and the three *ALT7* disruptants (T1 to T3) and one ectopic transformant (T4) using the primer pairs hphF/hphR (C)-a, ALT7inF/ALT7inR (C)-b, and ALT7homoF/hph homo R (C)-c.

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 [23,31] shared only by LASS/Lag homologs is found in Alt7 and the amino acid motif necessary for ceramide synthase activity is conserved in the protein (Figure 1B). Predicted transmembrane domains of TLC-containing proteins are also found in Alt7 (Figure 1C) [23,24]. 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 *Asc1* in tomato plants, indicated the possibility that *ALT7* acts as a self-tolerance gene for AAL-toxin in the toxin-producing *A. alternata* 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

recombination (Figure 2A,2B). Transformation of the tomato pathotype As-27 protoplasts with the *ALT7* disruption vector resulted in colonies, which were able to grow on PDA plates containing hygromycin B. These colonies were further purified by single spore isolation and examined for homologous integration by PCR screening. The primer set hphF/hphR produced the expected 0.4-kb band from all of the mutants (Figure 2Ca), and the primer set ALT7inF/ALT7inR resulted in no amplified fragments from the T1, T2 and T3 mutants (Figure 2Cb), suggesting that *ALT7* was deleted by the homologous integration of the disruption vector. In contrast, the transformant T4 showed the wild-type amplification band, indicating ectopic integration. To confirm the *ALT7* disruption, the primer combination ALT7homoF/hphhomoR was used to detect the junctions between the recipient *ALT7* region and the integrated vector (Figure 2B). With this primer combination, PCR failed to produce DNA fragments in the wild-type strain. In contrast, these primers produced the expected 1.2-kb band in the T1, T2 and T3 mutants (Figure 2Cc). The deletion of *ALT7* in the As-27 strain through homologous integration of the disruption vector was confirmed in the three mutants, and the T1 strain was selected for further experiments as the *ΔALT7* mutant.

Phenotypic characterization of *ALT7*-targeted and –complimented 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 defects 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 *Asc1* and is located in the *ALT* cluster of the tomato pathotype of *A. alternate*, 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

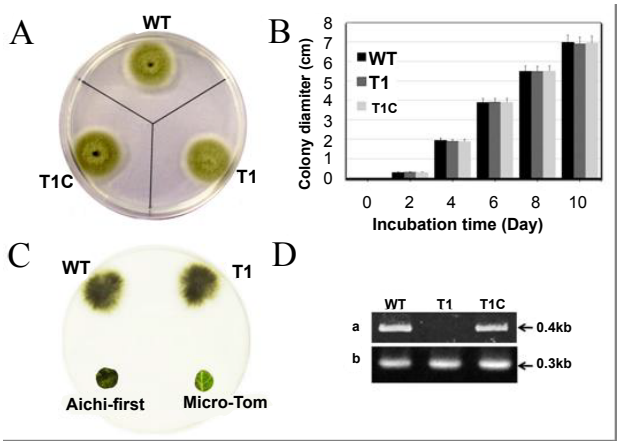

Figure 3: Phenotypic characterization of the *ALT7*-disrupted and *ALT7*-complimented strains. (A) Vegetative growth and colony morphology of wild type (WT), *ALT7*-disrupted (T1) and *ALT7*-complemented (T1C) strains on PDA. (B) The strains were grown on PDA at 25°C and colony diameters were recorded daily. The bars represent the means ± standard deviation of the diameters of three replicates. (C) Vegetative growth and colony morphology of the wild type (WT) and *ALT7*-disrupted (T1) strains on medium containing a high concentration of AAL-toxin. The high-toxin medium contains 50 μg/ml of AAL-toxin. Tomato leaf discs of susceptible (Aichi-first) and resistant (Micro-Tom) cultivars were also incubated on the medium as a control to confirm the activity of the toxin. Note that only the susceptible leaf disc showed necrosis. (D) Detection of *ALT7* transcripts in the transformants. Total RNA extracted from the wild type and the transformants cultured in 50 ml of PDB for 3 days with shaking was used as templates for RT-PCR. The primers ALT7inF and ALT7inR amplify an internal fragment (400 bp) of *ALT7*. Primers specific for β-tubulin were used as a positive control.

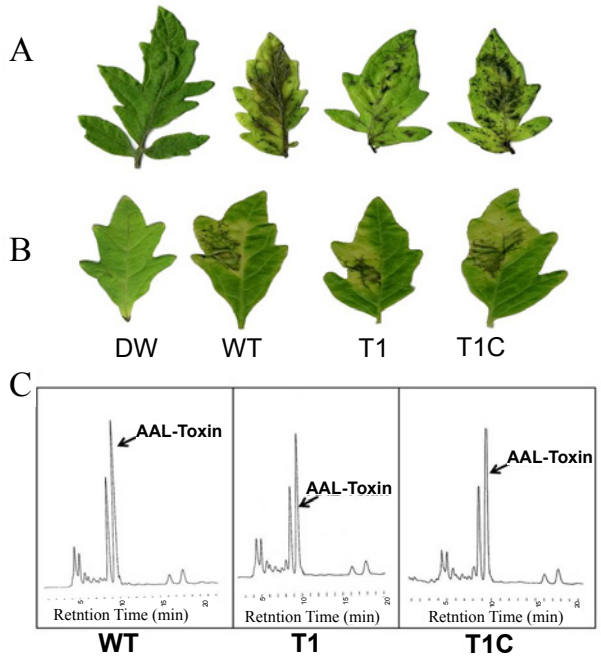

Figure 4: Pathogenicity and AAL-toxin production of the *ALT7*-disrupted strains. (A) Pathogenicity test by spore inoculation with *A. alternata* As-27 wild type (WT), *ALT7*-disrupted (T1) and *ALT7*-complemented (T1C) strains. The leaves were inoculated with a spore suspension (10^5 conidia/ml) and 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 extracted with $CH_3CN:H_2O$ (1:1, v/v). The extracts were cleaned using a Sep-Pak C18 cartridge column with $CH_3CN:H_2O$ (7:3, v/v) as a solvent. The OPA derivatization and HPLC analysis were described previously [12,28]. The peaks for AAL-toxin T_A are indicated by arrows.

genes with regard to cellular function, growth, morphology and toxin-protection of AAL-toxin-producing plant pathogens.

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References

1. Kohmoto K, Otani H, Tsuge T (1995) *Alternaria alternata* pathogens. In: Kohmoto K, Singh US, Singh RP, eds. Pathogenesis and Host Specificity in Plant Diseases: Histopathological Biochemical, Genetic and Molecular Bases, Vol. II. Eukaryotes. Oxford: Pergamon, 51-63.
2. Thomma BP (2003) *Alternaria* spp.: from general saprophyte to specific parasite. Mol Plant Pathol 4: 225-236.
3. Bezuidenhout **S**C, Gelderblom WCA, Gorst-Allman CP, Horak RM, Marasas WFO, et al. (1988) Structure elucidation of the fumonisins, mycotoxins from *Fusarium moniliforme*. J Chem Soc Chem Commun: 743-745.
4. Gilchrist DG, Grogan RG (1976) Production and nature of a host-specific toxin from *Alternaria alternata* f. sp. *Lycopersici*. Phytopathology 66: 165-171.
5. Kohmoto K, Otani H, Kodama M, Nishimura S (1989) Host-recognition: can accessibility to fungal invasion be induced by host-specific toxins without necessitating necrotic cell death? In: Graniti A, Durbin RD, Ballio A, eds. Phytotoxins and plant pathogensis. Springer, Berlin Heidelberg New York, 249-265.
6. Peever TL, Su G, Carpenter-Boggs L, Timmer LW (2004) Molecular systematics of citrus-associated *Alternaria* species. Mycologia 96: 119-134.
7. Gilchrist DG, Bostock RM, Wang H (1995) Sphingosine related mycotoxins in plant and animal disease. Can J Bot 73: S459-S467.
8. Spassieva SD, Markham JE, Hille J (2002) The plant disease resistance gene *Asc-1* prevents disruption of sphingolipid metabolism during AAL-toxin-induced programmed cell death. Plant Journal 32: 561-572.
9. Wang W, Jones C, Ciacci-Zanella J, Holt T, Gilchrist DG, et al. (1996) Fumonisins and *Alternaria alternata lycopersici* toxins: sphinganine analog mycotoxins induce apoptosis in monkey kidney cells. Proc Natl Acad Sci USA 93: 3461-3465.
10. Akamatsu H, Itoh Y, Kodama M, Otani H, Kohmoto K (1997) AAL-toxin-deficient mutants of *Alternaria alternata* tomato pathotype by restriction enzyme-mediated integration. Phytopathology 87: 967-972.
11. Brandwagt BF, Mesbah LA, Takken FL, Laurent PL, Kneppers TJ, et al. (2000) A longevity assurance gene homolog of tomato mediates resistance to *Alternaria alternata* f. sp. *lycopersici* toxins and fumonisin B1. Proc Natl Acad Sci 97: 4961-4966.
12. Yamagishi D, Akamatsu H, Otani H, Kodama M (2006) Pathological evaluation of host-specific AAL-toxins and fumonisin mycotoxins produced by *Alternaria* and *Fusarium* species. J Gen Plant Pathol 72: 323-327.
13. Fox EM, Howlett BJ (2008) Secondary metabolism: regulation and role in fungal biology. Curr Opin Microbiol 11: 481-487.
14. Keller N (2011) The fungal treasure chest: Spore origins? Fungal Biol Rev 25: 73-77.
15. Hoffmeister D, Keller NP (2006) Natural products of filamentous fungi: enzymes, genes, and their regulation, Nat Prod Rep 24: 393-416.
16. Walton JD (2000) Horizontal gene transfer and the evolution of secondary metabolite gene clusters in fungi: an hypothesis. Fungal Genet Biol 30: 167-171.
17. Akagi Y, Akamatsu H, Otani H, Kodama M (2009) Horizontal chromosome transfer: a mechanism for the evolution and differentiation of a plant pathogenic fungus. Eukaryot Cell 8: 1732-1738.
18. Akagi Y, Taga M, Yamamoto M, Tsuge T, Fukumasa-Nakai Y, et al. (2009) Chromosome constitution of hybrid strains constructed by protoplast fusion between the tomato and strawberry pathotypes of *Alternaria alternata*. J Gen Plant Pathol 75: 101-109.
19. Akamatsu H, Otani H, Kodama M (2003) Characterization of a gene cluster for host-specific AAL-toxin biosynthesis in the tomato pathotype of *Alternaria alternata*. Fungal Genet Newsl 50: 355.
20. Proctor RH, Brown DW, Plattner RD, Desjardins AE (2003) Coexpression of 15 contiguous genes delineates a fumonisin biosynthetic gene cluster in *Gibberella moniliformis*. Fungal Genet Biol 38: 237-249.
21. D'mello NP, Childress AM, Franklin DS, Kale SP, Pinswasdi C, et al. (1994) Cloning and characterization of *LAG*, a longevity-assurance gene in yeast. J Biol Chem 269: 15451-15459.
22. Mullen TD, Jenkins RW, Clarke CJ, Bielawski J, Hannun YA, et al. (2011) Ceramide synthase-dependent ceramide generation and programmed cell death: involvement of salvage pathway in regulating postmitochondrial events. J Biol Chem 286: 15929-15942.
23. Spassieva S, Seo JG, Jiang JC, Bielawski J, Alvarez-Vasquez F, et al. (2006) Necessary role for the Lag1p motif in (dihydro) ceramide synthase activity. J Biol Chem 281: 33931-33938.
24. Winter E, Ponting CP (2002) TRAM, LAG1 and CLN8: members of a novel family of lipid-sensing domains? Trends Biochem Sci 27: 381-383.
25. Schorling S, Vallee B, Barz WP, Riezman H, Oesterhelt D (2001) Lag1p and Lac1p are essential for the acyl-CoA-dependent ceramide synthase reaction in *Saccharomyces cerevisae*. Mol Biol Cell 12: 3417-3427.

26. Kuwayama H, Obara S, Morio T, Katoh M, Urushihara H, et al. (2002) PCR-mediated generation of a gene disruption construct without the use of DNA ligase and plasmid vectors. Nucleic Acids Res 30: E2.

27. Inoue I, Namiki F, Tsuge T (2002) Plant colonization by the vascular wilt fungus *Fusarium oxysporum* requires *FOW1*, a gene encoding a mitochondrial protein. Plant Cell 14: 1869-1883.

28. Kodama M, Otani H, Kohmoto K (1995) A rapid and sensitive procedure for the quantitative detection of AL-toxin by fluorescence derivatization and separation by high performance liquid chromatography. Ann Phytopathol Soc Jpn 61: 477-480.

29. Li S, Du L, Yuen G, Harris SD (2006) Distinct ceramide synthases regulate polarized growth in the filamentous fungus *Aspergillus nidulans*. Mol Biol Cell 17: 1218-1227.

30. Rittenour WR, Chen M, Cahoon EB, Harris SD (2011) Control of glucosylceramide production and morphogenesis by the Bar1 ceramide synthase in *Fusarium graminearum*. PLoS ONE 6: e19385.

31. Jiang JC, Kirchman PA, Zagulski M, Hunt J, Jazwinski SM (1998) Homologs of the yeast longevity gene *LAG1* in *Caenorhabditis elegans* and human. Genome Res 8: 1259-1272.

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