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Alternariol as virulence and colonization factor of *Alternaria alternata* during plant infection

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Summary

The filamentous fungus *Alternaria alternata* is a potent producer of many toxic secondary metabolites, which contaminate food and feed. The most prominent one is the polyketide-derived alternariol (AOH) and its derivative alternariol monomethyl ether (AME). Here, we identified the gene cluster for the biosynthesis of AOH and AME by CRISPR/Cas9-mediated gene inactivation of several biosynthesis genes in *A. alternata* and heterologous expression of the gene cluster in *Aspergillus oryzae*. The 15 kb-spanning gene cluster consists of a polyketide synthase gene, *pksI*, an O-methyltransferase, *omtI*, a FAD-dependent monooxygenase, *moxI*, a short chain dehydrogenase, *sdrI,* a putative extradiol dioxygenase, *doxI* and a transcription factor gene, *aohR*. Heterologous expression of PksI in *A. oryzae* was sufficient for AOH biosynthesis. Co-expression of PksI with different tailoring enzymes resulted in AME, 4-hydroxy-alternariol monomethyl ether (4-OH-AME), altenusin (ALN) and altenuene (ALT). Hence, the AOH cluster is responsible for the production of at least five different compounds. Deletion of the transcription factor gene *aohR* led to reduced expression of *pksI* and delayed AOH production, while overexpression led to increased expression of *pksI* and production of AOH. The *pksI*-deletion strain displayed reduced virulence on tomato, citrus and apple suggesting AOH and the derivatives as virulence and colonization factors.

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Introduction

More than 300 *Alternaria* species have been described to cause diseases in more than 400 plant species (Meena *et al.*, 2017; Pinto and Patriarca, 2017; Rychlik *et al.*, 2018). *A. alternata* alone infects already more than 100 plant species, and therefore, is an economically important food and feed contaminating fungus (Lee *et al.*, 2015). Although *A. alternata* generally can colonize different plants, there are also species-specific pathotypes that produce host selective toxins upon infection (Tsuge *et al.*, 2013; Cho, 2015). In addition to crop loss through plant infections, *A. alternata* causes tremendous damage in agriculture due to postharvest decay and contamination of food and feed with mycotoxins (Garganese *et al.*, 2016). At the beginning of the colonization when the fungal mycelium is still not visible and the decomposition of the material is still in its beginning, the products may be consumed and then cause severe health problems, because of the mycotoxins produced by the fungus. Several *A. alternata* secondary metabolites are classified as mycotoxins and have been evaluated by the European Food Safety Authority (EFSA) as potentially harmful to human health (EFSA, 2011). These include the mycotoxins alternariol (AOH), alternariol monomethyl ether (AME) and altertoxin I and II (Jarolim *et al.*, 2017). The health problems are usually not acute toxic but rather long-term effects, which may cause several mycotoxicoses, and in the worst-case end with cancer. The relation of such severe diseases to the consumption of intoxicated food are usually difficult, but likely the treatment of the diseases is very costly. In addition to *A. alternata* being a potent producer of secondary metabolites, it is known as one of the major causes of fungal allergies in human and animals (Breitenbach and Simon-Nobbe, 2002; Babiceanu *et al.*, 2013; Hayes *et al.*, 2018).

The possibility that *A. alternata* food contamination may be correlated to increased cases of esophageal cancer came from a study in Linxian, China (Dong *et al*., 1987; Zhen *et al*., 1991; Liu *et al*., 1991). It was shown that: (i) AME and AOH might cause cell mutagenicity and transformation, (ii) AME and AOH could combine with the DNA isolated from human fetal esophageal epithelium, activate the oncogens, c-H-ras and c-mys, and promote proliferation of human fetal esophageal epithelium *in vitro*, (iii) squamous cell carcinoma of the fetal esophagus could be induced by AOH. There are numerous studies showing the teratogenic and fetotoxic potential of *Alternaria* for mice (Pero *et al*., 1973; Pollock *et al*., 1982), and liver and kidney damage in rats was observed after feeding with *A. alternata* for 28 days (Combina *et al.,* 1999). Crude culture extracts of *Alternaria* were also mutagenic in the Ames test (Schrader *et al*., 2001). AOH has also been reported to possess cytotoxic, genotoxic and mutagenic properties *in vitro* and acts as a poison of topoisomerases (Fehr *et al*., 2009). Lehmann *et al.* (2006) discovered an estrogenic potential, an effect on cell proliferation and a genotoxic effect of AOH in cultured mammalian cells. AOH replaced E2 from isolated human estrogen receptors α and β and increased the level of alkaline phosphatase (ALP) mRNA and the enzymatic activity of ALP in a human endometrial adenocarcinoma cell line (Ishikawa cells). The estrogenicity of AOH was about 0.01% of E2. The effects in Ishikawa cells were reversed by the ER antagonist ICI 182,780. Analysis of cell proliferation by flow cytometry and microscopy of Ishikawa and Chinese hamster V79 cells revealed that AOH inhibited cell proliferation by interference with the cell cycle (Lehmann *et al*., 2006).

Despite the numerous studies on the toxicity of AOH toward animal cells, there are yet no official regulations about the concentrations tolerable in food and feed. In comparison to the numerous studies of AOH effects in animals, little is known about the role of AOH during the colonization process of plants. Approximately 70 phytotoxins are known to be produced by the genus *Alternaria*, some are host specific and some are not (EFSA, 2011; Escrivá *et al*., 2017). The latter affect a broad spectrum of plant species, have in general mild phytotoxic effects but support the colonization process by compensating the plant hypersensitive response (Stoll *et al*., 2014; Touhami *et al*., 2018), inhibit enzymatic reactions within plant tissue or lead to plant cell death and necrosis. Thus, they are not essential for successful infections, but they pave the way for establishing disease symptoms. Non-host-specific phytotoxins are mostly produced by saprophytic species, whereas host-specific-phytotoxins are produced by pathogenic isolates.

Here, we unraveled the biosynthetic pathway for AOH biosynthesis and show that a single gene cluster is responsible for the production of at least five different secondary metabolites. We present evidence that AOH facilitates growth of *A. alternata* on various fruits and leaves during infection.

Results

Analysis of the AOH gene cluster and synteny in other Alternaria *species*

To identify the AOH producing gene cluster in *A. alternata*, the genomic and protein sequence of Pks19 from *Parastagonospora nodorum,* which was shown to be responsible for AOH production in this fungus was used to search the *Alternaria* genome database (http://alternaria.vbi.vt.edu/index.html) (Chooi *et al*., 2015; Dang *et al*., 2015). *A. alternata* contains 10 different *pks* genes (Saha *et al*., 2012). PksI from *A. alternata* displayed 85% identical amino acids compared to *P. nodorum* Pks19*,* suggesting that PksI as a good candidate for AOH biosynthesis in *A. alternata*. The *pksI* gene is comprised of 5796 bp with three predicted introns of 49, 394 and 61 bp respectively. The introns were confirmed by synthesis and sequencing of cDNA. The derived protein consists of 1763 amino acids and an estimated molecular mass of 192.06 kDa. PksI is a typical non-reducing polyketide synthase that contains the minimum set of domains with a ketoacyl synthase (KS), an acyltransferase (AT) and an acyl carrier protein (ACP). In the vicinity of *pksI*, four genes, coding for enzymes typically involved in the modification of secondary metabolites, were identified (Fig. 1). In addition, the cluster contains a putative GAL4-like $Zn(II)_2Cys_6$ transcription factor encoding gene, named *aohR*. The four tailoring enzymes display similarity to an O-methyl transferase (*omtI*), a mono oxygenase (*moxI*), a short chain dehydrogenase (*sdrI*) and an estradiol dioxygenase (*doxI*) respectively. OmtI consists of 379 amino acids, MoxI of 385, SdrI of 230 and DoxI of 325 amino acids. The introns were confirmed by sequencing of corresponding cDNAs. The putative transcription factor was named AohR and consists of 588 amino acids.

In comparison with the genes of the cluster in *P. nodorum* the one in *A. alternata* lacks one O-methyltransferase, whereas that in *P. nodorum* lacks the mono oxygenase. Synteny analysis of the *A. alternata* gene cluster with other *Alternaria* species revealed that it is present in all sequenced strains except for *A. brassicicola* that probably lost the cluster in a deletion event since the neighboring genes of the cluster are still present (Fig. 1).

The polyketide synthase PksI catalyzes AOH formation in A. alternata

To analyze whether the *pksI* gene cluster of *A. alternata* is responsible for the production of AOH, we inactivated the *pksI* gene using the recently established CRISPR/Cas9

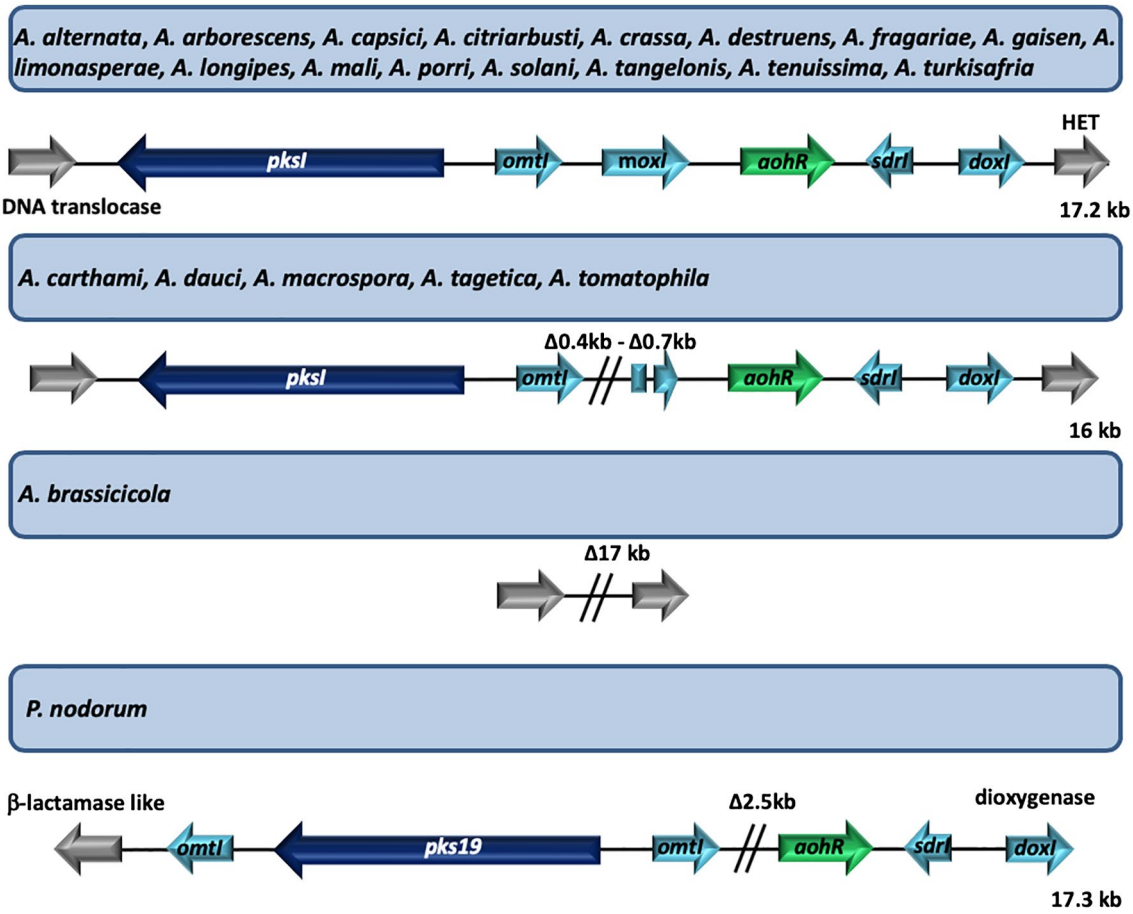

Fig. 1. Analysis of the AOH gene cluster and syntheny in *P. nodorum* and several *Alternaria* species. The cluster for AOH biosynthesis in many Alternaria species contains one pks (*pksI*), four tailoring enzymes (O-methyl transferase *omtI*, mono oxygenase *moxI,* short chain dehydrogenase like protein, *sdrI* and an estradiol dioxygenase, *doxI*) and a Gal4-like transcription factor. Some species are lacking the monooxygenase. *A. brassicicola* lost the cluster completely. The cluster in *P. nodorum* contains an additional O-methyl transferase and lacks the monooxygenase.

KO technology (Wenderoth *et al*., 2017). The protospacer was derived from the 5′-end of the gene, 30 bp downstream of the start codon (Fig. 2A). The resulting plasmid pMW36 was transformed into the WT strain ATCC66981. From 28 transformants, three had completely lost the ability to produce AOH and its derivatives as shown in thin layer chromatography and through HPLC analyses (Fig. 2B and C). One deletion mutant was further analyzed. PCR analysis of the cluster revealed that almost the entire cluster was deleted during the knock-out event including the *pksI* gene, the O-methyl transferase, the mono oxygenase and the transcription factor gene. None of these genes could be amplified by PCR, whereas the neighboring genes amplified as in wild type. Hence, the deletion had an estimated size of ca. 14 kb (Fig. 2A). To prevent neighboring genes from being affected by the random deletions that occur using this method and make the screening for mutants more efficient, all following deletions via CRISPR/Cas9 were performed with two simultaneously transformed constructs in the uracil-auxotrophic strain SMW24 (Wenderoth *et al*., 2017). The protospacers targeted the genes at the 5′ and at the 3′ end in order to remove the region between them. In the case of *pksI* the protospacer was placed 298 bp upstream of the stop codon targeted by the construct pMW61. This would cause a 5443 bp deletion if transformed together with pMW36. The frequency of successful deletions was higher than in single target transformations and the frequency of random deletion events was lower.

As a further proof that PksI is responsible for AOH biosynthesis, we employed a heterologous expression system established in *A. oryzae* (Wasil *et al*., 2013). This system is very well suited for heterologous expression, because *A. oryzae* produces very low levels of endogenous secondary metabolites and no AOH. We did not find any orthologue of PksI or enzymes with high similarity to any of the tailoring enzymes from the PksI gene cluster in the genome of *A. oryzae*. Expression of PksI

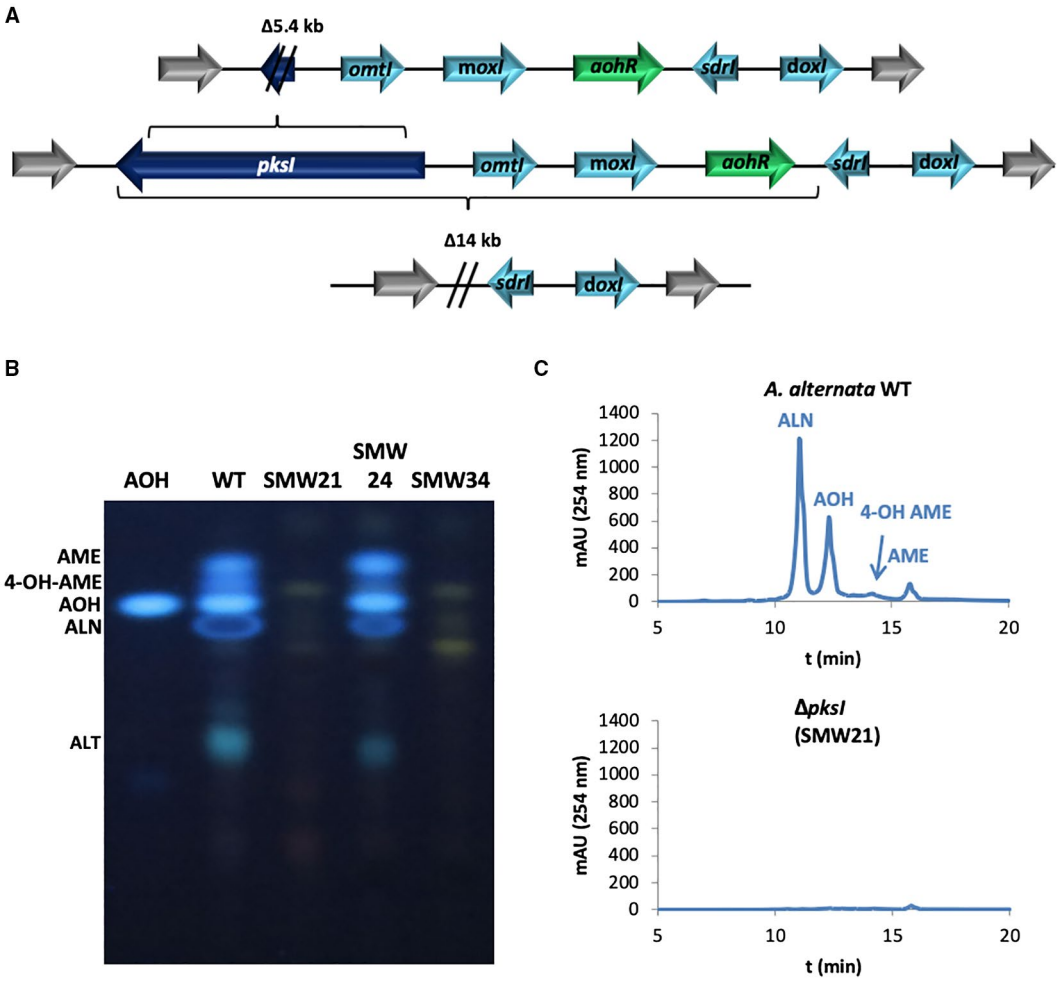

Fig. 2. *pksI* inactivation using the CRISPR/Cas9 technology. A. Scheme of the deletion events obtained using one (below = SMW21) or two (above = SMW34) protospacers. In comparison to the WT no AOH or the derivatives thereof could be detected in the extracts of SMW21 and SMW34 respectively which was verified by TLC (B) and HPLC (C).

alone in *A. oryzae* was sufficient to produce high amounts of AOH. The production was monitored with TLC, HPLC and LC-DAD-MS (Fig. 3 A–C). In the HPLC analysis a single peak with an elution time of 12.42 min occurred, suggesting a homogenous product. Next, we analyzed the role of the genes in the vicinity of *pksI*. The heterologous expression system in *A. oryzae* as well as the deletion of single tailoring enzyme genes in *A. alternata* allowed us to determine the function of those enzymes. Different combinations of tailoring enzymes along with PksI were introduced into *A. oryzae* with the constructs pMW28, 30, 31, 32 and 72 and the secondary metabolite profiles of the corresponding strains were analyzed. When the O-methyltransferase OmtI was expressed in combination with PksI (SMW15), the methyl ether of AOH, AME, was detected on TLCs and as a peak in the HPLC eluting at 15.89 min. AME was confirmed by LC-DAD-MS (Table 1). The intensity of the AOH band was lower as compared to the band from the strain which only expressed PksI, suggesting that AOH was converted to AME. The expression of the putative mono oxygenase MoxI (SMW19) led to the production of two new compounds. In the HPLC analysis one eluted at 11.11 min and the second at 13.82 min as a small peak. The LC-DAD-MS analysis revealed the chemical formula C15H14O6 for the first compound based on accurate masses, isotopic patterns and MS/MS spectra in positive and negative ion modes (Table 1). The predominant loss of CO_2 in the negative MS and MS/MS spectra was a hint for a carboxyl group suggesting altenusin (ALN) as candidate for the unknown peak (Fleck, 2013). A comparison of the *A. oryzae* samples with a commercially available ALN reference compound confirmed the peak with a retention time of 11.11 min as ALN (data not shown). Due to the predicted enzymatic activity of MoxI, the second unknown peak (13.82 min elution time) was suggested to be 4-hydroxy-alternariol monomethyl ether (4-OH-AME). For this second unknown peak, the LC-DAD-MS analysis revealed the chemical

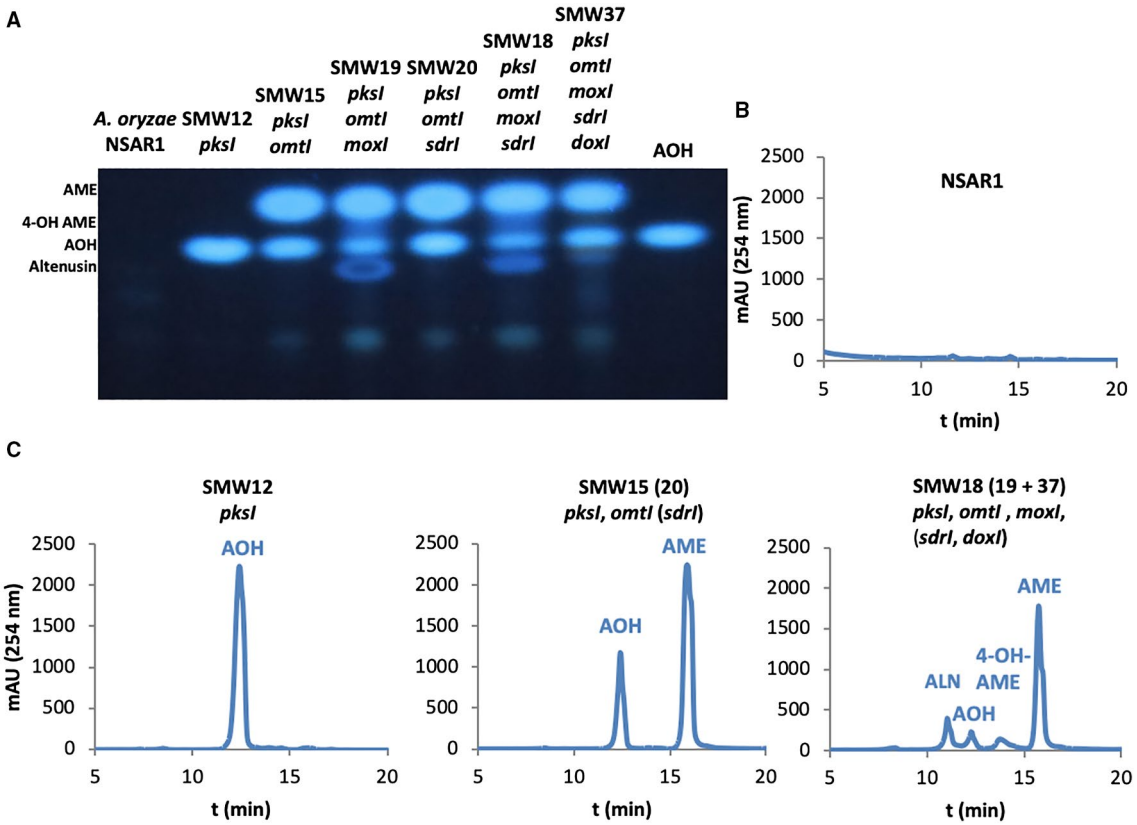

Fig. 3. Heterologous expression of *pksI* alone or in combination with different tailoring enzymes in *A. oryzae*.
A. TLC analysis of the different *A. oryzae* strains: NSAR1, SMW12 (*pksI*), SMW15 (*pksI* and *omtI*), SMW18 (*pksI*, *omtI*, *moxI* and *sdrI*), SMW19 (*pksI*, *omtI* and *moxI*), SMW20 (*pksI*, *omtI* and *sdrI*) and SMW37 (*pksI*, *omtI*, *moxI*, *sdrI* and *doxI*). The expression of *pksI* leads to AOH formation. Through additional expression of *omtI* AME can be detected in the extracts. The expression of *moxI* leads to the production of 4-OH-AME and Altenusin (ALN). Expression of *sdrI* and *doxI* did not produce any additional compounds.
B. HPLC analysis of the recipient *A. oryzae* strain NSAR1.
C. HPLC analyses of the *A. oryzae* strains.

formula $C_{15}H_{12}O_6$ based on accurate masses, isotopic patterns and MS/MS spectra in positive and negative ion modes (Table 1). This is in agreement with the elemental composition of 4-OH-AME. Furthermore, two main fragments, i.e. m/z 188 and m/z 272, were detected in the negative MS/MS. This confirms previous measurements, where the same two fragments were identified (Tiessen *et al.*, 2017). In addition, the unknown second peak eluted between AOH and AME at comparable LC conditions (Tiessen *et al.*, 2017). Taken together the data for the second unknown peak strongly suggest that it is 4-OH-AME. The deletion of *moxI* in *A. alternata* led to the loss of ALN and 4-OH-AME (Fig. 4).

Expression of *sdrI* neither together with all other enzymes nor in different combinations (strains SMW18, 20), led to the production of an additional product in *A. oryzae* (Fig. 3). To see if the gene has a function in AOH modification we targeted *sdrI* in *A. alternata* using two CRISPR/Cas9 constructs (pMW56/57) for deletion. The obtained mutant strain SMW32 was confirmed by sequencing and showed the expected 692 bp deletion within the *sdrI* gene. In contrast to the WT and parental strain SMW24 the deletion strain did not produce ALN (Fig. 4). Since the band appeared in *A. oryzae* whether *sdrI* was present or not during co-expression with *moxI*, it is most likely that a promiscuous enzyme present in *A. oryzae* takes over the role of *sdrI* to convert 4-OH-AME to ALN.

Co-expression of the putative dioxygenase, *doxI,* was performed using a second plasmid (pMW72) that was transformed into the *A. oryzae* strain SMW18 already expressing *pksI*, *omtI*, *moxI* and *sdrI*. Incubation of the resulting transformants (Strain SMW37) on production medium did not lead to the production of an additional compound. However, the bands of 4-OH-AME and ALN were weaker than in the parental strain. In parallel, the gene was also deleted in *A. alternata* SMW24 using the constructs pMW76/77. The obtained mutant showed the expected deletion of 755 bp between the two protospacers. In comparison to the parental and the wild-type strain after 7 days of growth the mutant lacked a band in the

Table 1. Measured and theoretical mass/charge ratios, mass errors and selected fragment ions of the secondary metabolites variably observed in the LC-DAD-MS analyses of the *A. oryzae* samples.

Secondary metabolite	MS mode	Measured mass ($M + H^+$ or $M - H^+$) [m/z]	Theoretical mass ($M + H^+$ or $M - H^+$) [m/z]	Mass error [ppm]	Selected fragment ions [m/z]
Alternariol (AOH)	Positive mode	259.0602	259.0601	0.5	244, 241, 213, 185, 128
	Negative mode	257.0458	257.0456	0.9	257, 215, 213, 212, 159, 147
Alternariol monomethyl ether (AME)	Positive mode	273.0767	273.0758	3.4	258, 230, 185, 184, 169, 139
	Negative mode	271.0612	271.0612	−0.1	256, 255, 228, 227, 213, 211, 183, 155
Altenusin (ALN)	Positive mode	291.0863	291.0863	−0.2	273, 255, 227, 199, 139, 128
	Negative mode	289.0720	289.0718	1.0	271, 245, 230, 229, 161, 160, 159, 146
4-Hydroxy-alternariol monomethyl ether (4-OH-AME)[a]	Positive mode	289.0710	289.0707	1.1	271, 243, 215, 200, 144, 115
	Negative mode	287.0564	287.0561	1.1	272, 257, 229, 188, 187, 185

[a]4-OH-AME structure is still preliminary.

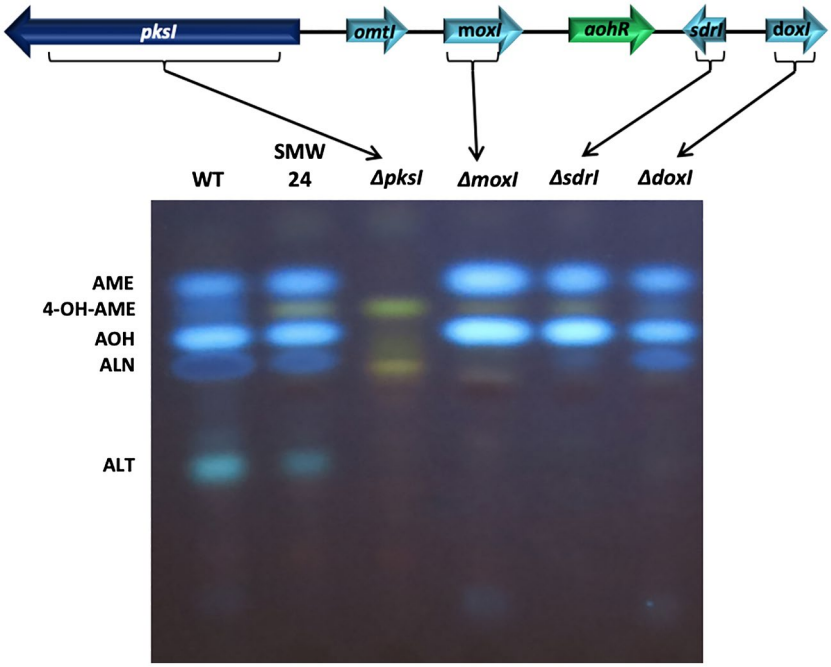

Fig. 4. Deletion analysis of the AOH gene cluster. The TLC analysis shows the metabolite profile of the four deletion strains illustrated in the scheme above. Strains: WT, SMW24, SMW34 (Δ*pksI*), SMW36 (Δ*moxI*), SMW32 (Δ*sdrI*) and SMW52 (Δ*doxI*). Deletions were done in the strain SMW24. Deletion of *pksI* leads to the loss of AOH-production and all detectable derivatives. Deletion of *moxI* leads to the loss of 4-OH-AME, ALN and ALT. *sdrI*-deletion leads to the loss of ALN and ALT and *doxI*-deletion to the loss of ALT production.

TLC analysis. This band runs lower than the other derivatives and shows in contrast a turquoise color. Through comparison with a commercial standard the substance could be identified as altenuene (ALT) (Fig. 4).

The Gal4-like transcription factor AohR enhances the expression of the pksI*-gene cluster*

The *pksI* cluster contains a putative transcription factor. The transcription factor AohR contains two specific domains. One GAL4-like $Zn(II)_2Cys_6$ (or C6 zinc) binuclear cluster DNA-binding domain and a fungal-specific transcription factor domain. The ORF has a size of 1767 nucleotides with no predicted introns. The protein is comprised of 588 amino acids and has a predicted molecular mass of 65.51 kDa. In order to investigate its potential role in the regulation of the cluster genes, we used CRISPR/Cas9 knock-out constructs in order to delete the gene from the cluster. Two protospacers were chosen to remove a 1094 bp fragment (plasmids pMW53 and pMW55) (Fig. 5A) of the gene which leads to the deletion of the essential DNA-binding domain as well as a frameshift causing premature stop codons. All the strains with confirmed deletions of *aohR* showed delayed and reduced production of AOH and its derivatives. In the parental strain SMW24 the production

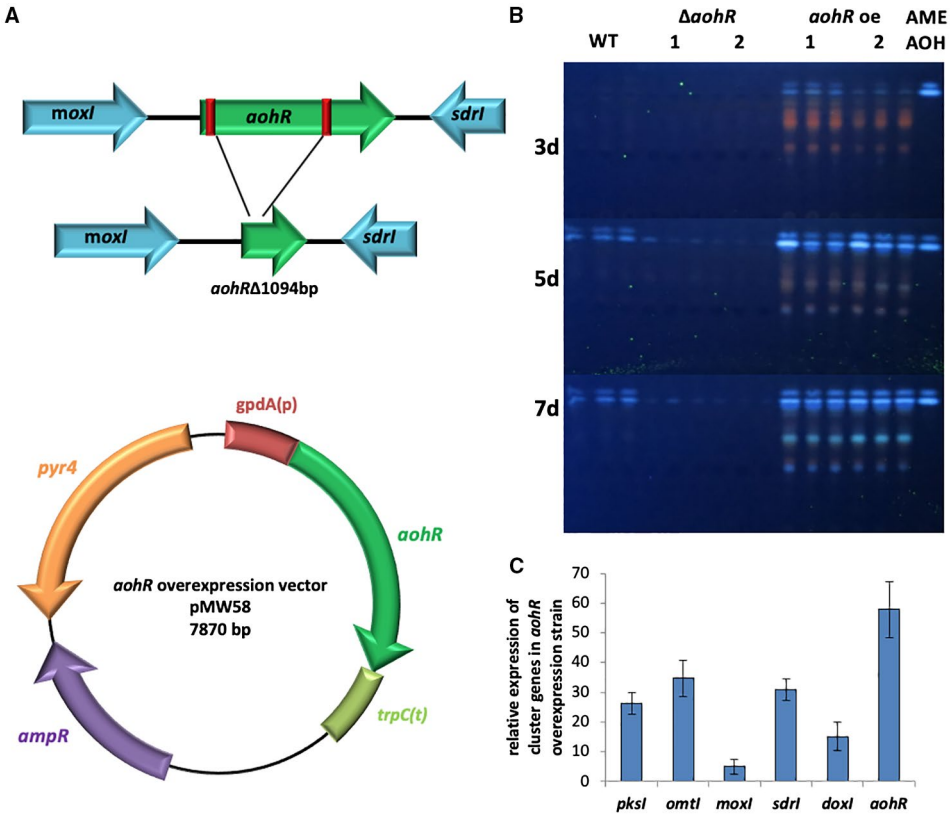

Fig. 5. AohR is a Gal4-like transcription factor that regulates AOH-cluster genes.
A. Scheme illustrating the deletion event in SMW24 where a 1094 bp deletion occurred between the protospacers highlighted in red. The plasmid pMW58 used for overexpression is shown below.
B. TLC analysis of the *aohR*-deletion strain SMW31 and the *aohR*-overexpression strain SMW33. Three samples of each strain were analyzed after 3, 5 and 7 days of growth. SMW31 shows a delayed and weak production of AOH compared to the parental strain SMW24 while in SMW33 the production starts already at 3 days of incubation and after 5 days all known AOH derivatives were detected.
C. Expression analysis of the genes of the AOH cluster in the *aohR*-overexpression strain SMW33. All genes showed an enhanced expression reaching from fivefold (*moxI*) to 35-fold (*omtI*). The overexpression of aohR itself was 58-fold higher than in the parental strain.

of AOH and AME began after 5 days on solid mCDB medium. In the mutant strains only weak AOH production was observed after 5 days with no enhancement or additional production of AME after 7 days (Fig. 5B). These results suggest a basal transcription of the cluster genes in the absence of AohR, but a stimulation of the expression in the presence of the transcription factor. To further confirm this hypothesis, we tested the effect of overexpression of *aohR* on the AOH production. To achieve overexpression, *aohR* was placed under the control of the strong constitutive *gpdA*-promoter from *A. nidulans* which is also functional in *A. alternata* (Fig. 5A). Of the transformants that were positively tested for integration of the overexpression cassette, two were chosen for analysis of AOH production. After 3 days on solid medium AOH and AME production could be observed and after 5 days ALN and ALT appeared. After 7 days, the AOH production reached its maximum together with the other so far known AOH derivatives. The activating role of AohR for the expression of *pksI* could further be proven by quantitative real time PCR. AOH production does not occur early in mycelium that is incubated in stationary liquid medium. Overexpression of *aohR* led up to 26-fold increase in *pksI* expression under those conditions. The genes of the tailoring enzymes showed also increased expressions from 5 to 35-fold (Fig. 5C).

AOH as pathogenicity factor

Secondary metabolites often play important roles in pathogenicity. However, in the case of AOH, only the toxic effect on mammalian cells has been well studied, because AOH is a very frequent mycotoxin in food and feed. There are only few studies on its effects on plant cells and only one report for a potential role in plant pathogenicity (Furutani *et al*., 2004; Graf *et al*., 2012; Meena *et al*., 2017). Since *A. alternata* is known to have a broad host range and often infects crop plants and fruits before harvest, we performed infection assays on different targets

of *A. alternata*. Tomatoes, apples and citrus fruits were infected with a spore suspension and incubated for 7 days at 20°C, 25°C and 28°C to form lesions. The lesions were afterwards measured in diameter and area. The lesions on tomatoes infected with the *pksI*-deletion strain SMW21 were reduced by 52% in area compared to the lesions caused by the WT strain when incubated at 28°C. When an AOH (50 µg) solution was added to the fruits that were infected with the knock-out strain, the lesions had nearly the same size as those caused by the WT strain implicating an involvement of this metabolite in the colonization process (Fig. 6A and B). Next, we tested whether AOH production was stimulated during growth on tomatoes. To this end, *A. alternata* was cultured in liquid medium in the presence of tomato pieces. After 4 days of growth, the control culture did not produce detectable amounts of AOH and AME, whereas both compounds were detected in the extracts of mycelium grown with tomato pieces (Fig.

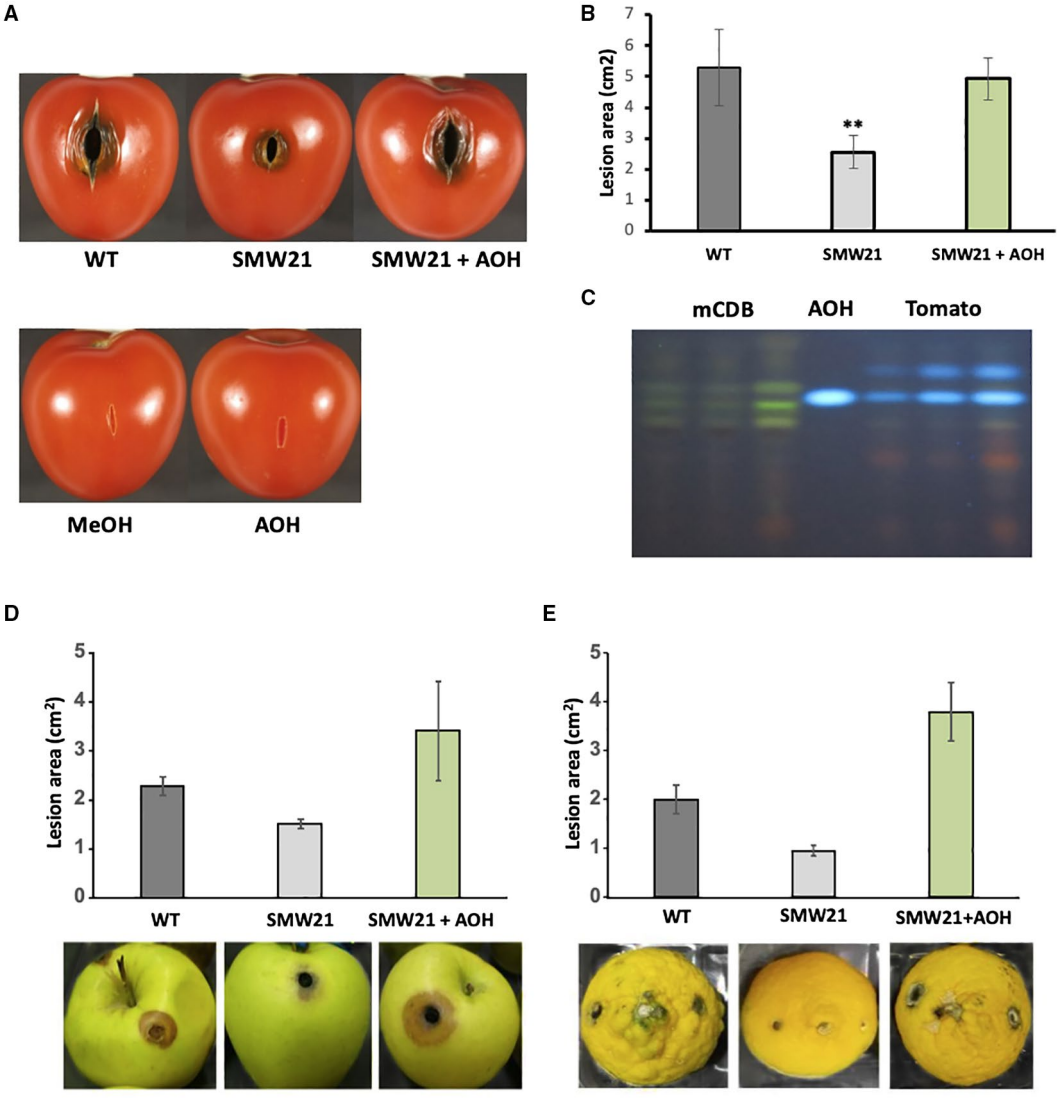

Fig. 6. AOH as a virulence factor in plant infection on tomato, citrus and apple.
A. Tomatoes were infected with *A. alternata* strains ATCC 66981, SMW21, or with SMW21 plus 10 µl of a 5 µg/µl AOH solution. Control tomatoes were treated with methanol (MeOH) or AOH. Tomatoes were then incubated for 7 days at 28°C in darkness. The lesions caused by the deletion strains were reduced in size by 63% and could be restored to wild-type levels by addition of AOH.
B. Quantification of the lesion areas. Nine lesions per strain were analyzed. The error bar indicates the standard deviation. The two asterisks mean values are significantly different in deletion strains (unpaired t-test; $p < 0.01$).
C. TLC analysis of extracts from *A. alternata* strain ATCC 66981 incubated in liquid mCDB and liquid mCDB with tomato fragments. Three replicates for each condition were incubated for 4 days at 28°C.
D. Infection of apples.
E. Citrus with strain ATCC 66981, SMW21 and SMW21 supplemented with AOH (50 µg) inoculated in a 0.5 cm diameter hole. In apples, the lesion areas were reduced by 34%, in citrus by 53% compared to wild type. Addition of AOH to SMW21 infection sites increased the lesion areas by 49% and 90% respectively.

6C). Similar results as with tomatoes were obtained in infection assays with apple and citrus fruits. The lesion area was 34% reduced on apples and 53% on citrus fruits. The addition of AOH to the AOH-deficient strains increased virulence even above WT levels by 49% for apples and 90% for citrus fruits (Fig. 6D and E).

Discussion

AOH is a potent mycotoxin with various cytotoxic effects. The biosynthetic pathway for AOH and its derivatives was only partly known and although there was some evidence about a role in pathogenicity, direct evidence was missing. Here, we show that the *pksI* gene cluster of *A. alternata* is responsible for the production of the compound and several derivatives thereof. This is in agreement with the results obtained for *pks19* in *P. nodorum,* although the *moxI* gene is missing in the cluster of *P. nodorum* in comparison to *A. alternata* (Chooi *et al*., 2015). Originally, in *A. alternata* PksJ was assigned the function of AOH biosynthesis (Saha *et al*., 2012). This was based on downregulation of several *pks* gene clusters using RNAi technology and expression analyses and obviously led to a wrong conclusion. Here, we used the CRISPR/Cas9 technology for clean gene inactivation in *A. alternata* and a heterologous expression system to express the AOH gene cluster in *A. oryzae*. AOH is further converted in *A. alternata* by the methyl transferase OmtI to AME. Addition of the monooxygenase MoxI resulted in the production of ALN (Fig. 7). AME and ALN differ in one hydroxyl group at position 4 and the opening at the lactone ring creating a carboxylic acid residue. The hydroxylation reaction at position 4 is probably catalyzed by the monooxygenase MoxI resulting in 4-hydroxy-AME as intermediate that can also be detected in *A. alternata*. The question remained how the ring opening of 4-hydroxy-AME could occur. Although SdrI is one candidate, expression of MoxI alone in *A. oryzae* was sufficient for ALN production from AME. On the other hand, deletion of *sdrI* in *A. alternata* caused a loss of ALN production. However, it is also plausible that the synthesis pathway branches from PksI by reduction before the lactonization occurs (Sun *et al*., 2012). In this case, OmtI and MoxI would modify this intermediate in the same way as AOH that would lead to ALN in the end. However, the suggested intermediates were not detected in the extracts. The formation of ALN in *A. oryzae* without the presence of SdrI could be explained by an unspecific reaction of a reductase present in the heterologous host but not in *A. alternata*. The subsequent formation of ALT from ALN is most likely started by the putative estradiol dioxygenase DoxI. These enzymes perform ring openings next to the two hydroxyl groups in an aromatic ring. ALT could be formed by rotation of the C-ring and a following lactonization. This would explain the changed positions of the two hydroxy groups from 3, 4 to 2, 3 and also the methyl group being placed at position 4a instead of 1. The initial ring opening by an estradiol dioxygenase could initiate these rearrangements. However, since the reaction could not be reproduced in the heterologous expression system the involvement of additional biosynthetic enzymes in *A. alternata* is likely since further reduction steps are necessary to form ALT from the possible intermediates. The analysis of the surrounding genes of the AOH producing gene cluster did not lead to the discovery of potential biosynthetic genes that could be involved in further processing of the molecule. In summary, we resolved the biosynthetic pathway for AOH and its derivatives and show that one gene cluster is responsible for the production of at least five different compounds. The information can now be used to develop lab-on-a-chip PCR-based detection methods in food and feed for monitoring AOH contaminations in very early stages (Chaumpluk *et al*., 2016).

Another important finding of this paper is the role of AOH and its derivatives in pathogenicity. Secondary metabolites are well-known to play roles in virulence of animal and plant pathogenic fungi (Scharf *et al*., 2014). *A. alternata* species are able to produce host specific toxins to enable infection of certain plant species (Tsuge *et al*., 2013). A correlation between the reduction of AOH production in more resistant tomato genotype and a *hogA* mutant of *A. alternata* was taken as evidence for the role of AOH as virulence supporting colonization factor (Graf *et al*., 2012; Wojciechowska *et al*., 2014). Here, we present direct evidence that AOH supports colonization of tomatoes, apples and citrus fruits with *A. alternata.* The mechanism however, remains to be determined. Several effects of AOH on animal cells have been described. AOH induced DNA strand breaks in human colon carcinoma cells (HT29) (Fehr *et al*., 2009). Within 1 h of incubation, AOH significantly increased the rate of DNA strand breaks at concentrations $\geq$1 μM indicating substantial genotoxic potential. In a cell-free system, AOH was found to inhibit the catalytic activity of topoisomerase I at concentrations $\geq$50 μM. The catalytic activity of topoisomerase IIα and IIβ was significantly suppressed by AOH at $\geq$25 μM and $\geq$150 μM respectively indicating a preference of this mycotoxin toward the IIα isoform (Fehr *et al*., 2009). In HT29 cells, the inhibition of topoisomerase I and II activity was associated with the stabilization of the topoisomerase I- and II-DNA complexes. In summary, AOH was identified as a topoisomerase I and II poison which might cause or at least contribute to the impairment of DNA integrity in human colon carcinoma cells. These effects of AOH could also happen during plant colonization, although these mechanisms are probably not responsible for acute toxicity and the possible cause of necrosis by AOH, because the tissue cells are not dividing. On the

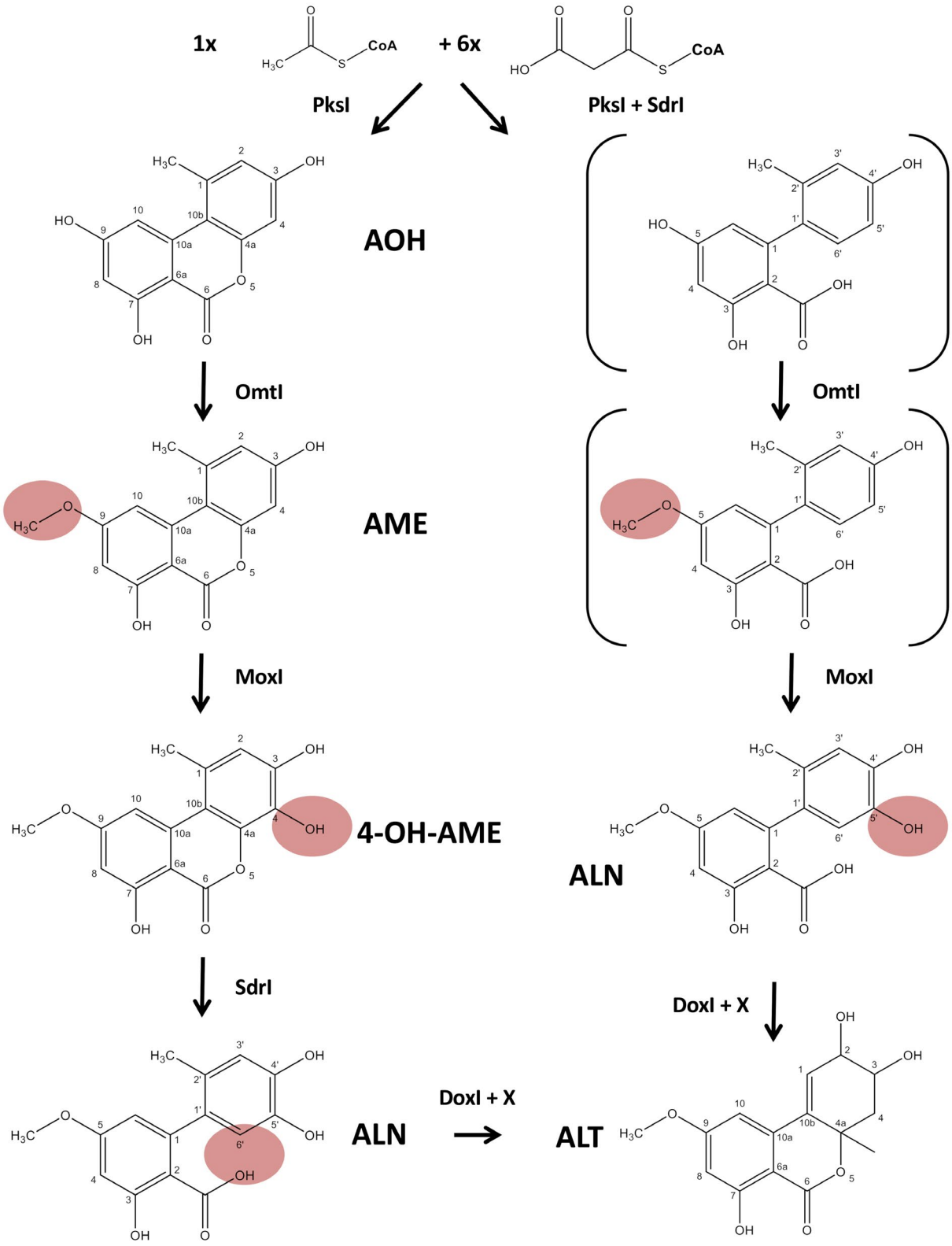

Fig. 7. Biosynthesis scheme for AOH, AME, 4OH-AME, ALN and ALT. The production starts with PksI through the assembly of one acetyl-CoA together with six malonyl-CoA to the heptaketide AOH. OmtI forms a methyl ether with the 9-hydroxy group resulting in AME. 4-OH-AME is formed by addition a hydroxyl group at position 4. Opening of the lactone ring is probably catalyzed by SdrI. An alternative pathway (molecules in brackets) starts with the reduction step of SdrI before the lactone is formed. Methylation and hydroxylation of this putative intermediate at position 5 and 5′ leads to the formation of ALN. The production of ALT probably starts with DoxI from ALN. [Colour figure can be viewed at wileyonlinelibrary.com]

other hand, AOH is able to induce cell death through the activation of the mitochondrial pathway of apoptosis in human colon carcinoma cells (Bensassi *et al*., 2012). In planta it was shown that AME is able to inhibit electron transport in the photosynthetic process in isolated spinach chloroplasts (Demuner *et al*., 2013). This suggests a combinatorial role of AOH and AME during plant infection by inhibition of photosynthesis in leaves and induction of cell death and thus facilitating the growth in different plant tissues. This may explain the broad host range since these mechanisms apply to all plants. The fact that we found induction of the production of AOH and AME by plant material is further evidence that that AOH and AME are indeed important virulence factors.

Another interesting aspect of the AOH gene cluster is its capability to produce ALN. ALN was reported to have antifungal activities and thus might play a role during infection by inhibiting the growth of other fungi on the substrate (Johann *et al*., 2012). Together with the synergistic action of combined mycotoxin pairs in a host regarding toxicity and virulence (Speijers and Speijers, 2004; Schulz *et al*., 2018), this suggests a complex interplay of different secondary metabolites during microbial competition within a habitat and colonization of different hosts.

Experimental Procedures

Strains, plasmids and culture conditions

A. alternata strain ATCC66981 was grown on mCDB (4% of glucose, 0.1% of yeast extract 0.1% of $NaNO_3$, 0,.025% of NH_4Cl, 0.1% of KH_2PO_4, 0.025% of KCl, 0.025% of NaCl, 0.05% of $MgSO_4$ ($7 \times H_2O$), 0.001% of $FeSO_4$, 0.001% $ZnSO_4$ and 1.5% of agar) at 28°C (180 rpm in liquid culture). *A. oryzae* strain NSAR1 was grown on mCD (1.5% of glucose, 0.4% of yeast extract, 0.1% of KH2PO4, 0.05% of $MgSO_4$ ($7 \times H_2O$), 0.001% of $FeSO_4$, $\times 7H_2O$, 1.5% of agar, pH 7) at 28°C. For metabolite expression all strains were incubated on MPY (2% of maltose, 1% of polypeptone, 0.5% of yeast extract, 0.5% of KH_2PO_4, 0.05% of $MgSO_4$ ($7 \times H_2O$), 1.5% of agar, pH7) at 28°C (180 rpm in liquid culture). All strains are listed in Table 2, oligonucleotides in Table 3 and plasmids are listed in Table 4.

Protoplast transformation of A. alternata

Fungal spores were harvested from a mCDB culture plate and inoculated into 100 ml of liquid mCDB for overnight cultivation at 28°C and 180 rpm. The mycelium was harvested by filtering, washed with 0.7 M of NaCl and digested in a Kitalase (Wako Chemicals) suspension (150 mg in 15 ml 0.7 M of NaCl) for 1 h with soft shaking at 100 rpm at 30°C. Protoplast quality and quantity were checked via microscopy. Protoplasts were separated from cell fragments by filtering through two layers of Miracloth and precipitated at 680 g for 10 min at room temperature. The Kitalase solution was discarded and the protoplasts were washed once with ice cold 0.7 M of NaCl and the centrifugation step was repeated. Afterwards, the protoplasts were resuspended in 100 µl of STC (1 M of sorbitol, 50 mM of $CaCl_2$ and 50 mM of Tris-HCl, pH 8). About 5–10 µg of Plasmid DNA were added to the protoplasts followed by a 10 min incubation on ice. DNA uptake was induced with a heat shock at 42°C for 5 min and, after a 5 min incubation step on ice, 800 µl 40% PEG (40% polyethylene glycol [PEG] 4000, 50 mM of Tris-HCl [pH 8] and 50 mM of $CaCl_2$) was added to the protoplasts, followed by 15 min incubation at room temperature. The suspension was mixed with 50 ml of warm regeneration medium and split into two petri dishes. After overnight incubation at 28°C the transformation plates were overlayed with 15 ml of warm regeneration medium

Table 2. Strains used in this study.

Strain	Genotype	Reference
A. alternata ATCC 66981	Wild type	Christopher B. Lawrence, Virginia Tech, Blacksburg, VA, USA
A. alternata SMW21	ATCC 66981 Δ*pksI*; Δ*omtI*; Δ*moxI*; Δ*aohR* after transformation with pMW36	This study
A. alternata SMW24	Δ*pyrG*	Wenderoth *et al.* (2017)
A. alternata SMW31	Δ*aohR* after transformation with pMW53 and pMW55	This study
A. alternata SMW32	SMW24 Δ*sdrI* after transformation with pMW56 and pMW57	This study
A. alternata SMW33	*gpdA*(p)::*aohR*::*trpC*(t)	This study
A. alternata SMW34	SMW24 Δ*pksI* after transformation with pMW36 and pMW61	This study
A. alternata SMW35	SMW34 transformed with *pyr4*	This study
A. alternata SMW36	SMW24 Δ*moxI* after transformation with pMW70 and pMW71	This study
A. alternata SMW52	SMW24 Δ*doxI* after transformation with pMW76 and pMW77	This study
A. oryzae NSAR1	*niaD*$^{-}$, *sC*$^{-}$, Δ*argB*, *adeA*$^{-}$	Russel J. Cox, Leibniz Universität Hannover
A. oryzae SMW12	*A. oryzae* NSAR1 transformed with pMW15	This study
A. oryzae SMW15	*A. oryzae* NSAR1 transformed with pMW28	This study
A. oryzae SMW18	*A. oryzae* NSAR1 transformed with pMW30	This study
A. oryzae SMW19	*A. oryzae* NSAR1 transformed with pMW31	This study
A. oryzae SMW20	*A. oryzae* NSAR1 transformed with pMW32	This study
A. oryzae SMW37	SMW18 transformed with pMW72	This study

Table 3. Oligonucleotides used in this study.

Oligonucleotide	Sequence 5′ → 3′
Crispr fw 2.0	GGTCATAGCTGTTTCCGCTGA
Crispr re 2.0	TGATTCTGCTGTCTCGGCTG
pksI Proto fw1	GTCCGTGAGGACGAAACGAGTAAGCTCGTCAGAGATACCTCAAGGGGATCGTTTTAGAGCTAGAAAT AGCAAGTTAAA
pksI 6bp re1	GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAGAGAGATCGGTGATGTCTGCTCAAGCG
pksI Proto fw2	GTCCGTGAGGACGAAACGAGTAAGCTCGTCTCCGAACACTCCGATTCTTCGTTTTAGAGCTAGAAATAGCAA GTTAAA
pksI 6bp re2	GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAGTCCGAACGGTGATGTCTGCTCAAGCG
moxI Proto fw1	GTCCGTGAGGACGAAACGAGTAAGCTCGTCGGAGTCGATGCCGGAGGAGAGTTTTAGAGCTAGAAATAGCAAGTTAAA
moxI 6bp re1	GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAGGGAGTCCGGTGATGTCTGCTCAAGCG
moxI Proto fw2	GTCCGTGAGGACGAAACGAGTAAGCTCGTCACATGCTCTCACGTGGTAAGGTTTTAGAGCTAGAAATAGCAAGTTAAA
moxI 6bp re2	GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAGACATGCCGGTGATGTCTGCTCAAGCG
sdrI Proto fw1	GTCCGTGAGGACGAAACGAGTAAGCTCGTCGTGGTCCTCATCCTCGGCTCGTTTTAGAGCTAGAAATAGCAAGTTAAA
sdrI 6bp re1	GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAGGTGGTCCGGTGATGTCTGCTCAAGCG
sdrI Proto fw2	GTCCGTGAGGACGAAACGAGTAAGCTCGTCTGGCAGGCCACTTTCGTGAAGTTTTAGAGCTAGAAATAGCAAGTTAAA
sdrI 6bp re2	GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAGTGGCAGCGGTGATGTCTGCTCAAGCG
doxI Proto fw1	GTCCGTGAGGACGAAACGAGTAAGCTCGTCCTACGACCACCCCATCTTCAGTTTTAGAGCTAGAAATAGCAAGTTAAA
doxI 6bp re1	GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAGCTACGACGGTGATGTCTGCTCAAGCG
doxI Proto fw2	GTCCGTGAGGACGAAACGAGTAAGCTCGTCTTCCAATCCAGAAGAACGCAGTTTTAGAGCTAGAAATAGCAAGTTAAA
doxI 6bp re2	GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAGTTCCAACGGTGATGTCTGCTCAAGCG
aohR Proto fw1	GTCCGTGAGGACGAAACGAGTAAGCTCGTCTCCGCAGTTTCACAGGCTGTGTTTTAGAGCTAGAAATAGCAAGTTAAA
aohR 6bp re1	GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAGTCCGCACGGTGATGTCTGCTCAAGCG
aohR Proto fw2	GTCCGTGAGGACGAAACGAGTAAGCTCGTCATCAACCTCGAGGAAACAGTGTTTTAGAGCTAGAAATAGCAAGTTAAA
aohR 6bp re2	GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAGATCAACCCGGTGATGTCTGCTCAAGCG
pksI ory fw	AAGAGTCAGTCAGTCTTAATATGATACGGGCCAAGGTCCT
pksI ory re	ATCGACTGACTGACTTTAATCTACTGAAGCAACTTCTTGAGATCT
omtI ory fw	CAAGATCCCAAAGTCAAAGGATGGTGGCCTACACTCTTATGG
omtI ory re	GACGTCATATAATCATACGGCTAAGCCAGTGTTGCACCAATG
omtI ory re2	GTTATGAACATGTTCCCTGGCTAAGCCAGTGTTGCACCAATG
moxI ory fw	GCTTGAGCAGACATCACCGGATGTCGACCAAGTCGTACCCT
moxI ory re1	GACGTCATATAATCATACGGCTACTGCACGGCAACAACCG
moxI ory re2	ATATCATCAATCATGACCGGCTACTGCACGGCAACAACCG
sdrI ory fw1	GCTTGAGCAGACATCACCGGATGGCTCCTGTGGTCCTCAT
sdrI ory fw2	ATTCCGCAGCTCGTCAAAGGATGGCTCCTGTGGTCCTCAT
sdrI ory re	GACGTCATATAATCATACGGTCACTTGAAAGAGGCATATCCCT
doxI ory fw	CAAGATCCCAAAGTCAAAGGATGCCCTTGGCGCTGTCGCC
doxI ory re	GACGTCATATAATCATACGGTCACGCTGTAGTCGGAAGTTCA
aohR oe fw	GCTTGAGCAGACATCACAGGATGGAAACTCACACAATCCGC
aohR oe re	TCAGTAACGTTAAGTTTAATCTAGACTAACAACACCAAGTCTC

containing HygB (80 μg ml^{-1}) and again incubated until colonies were formed.

Protoplast transformation of A. oryzae

A. oryzae strains were grown on mCD for 7 days at 28°C. Spores were harvested an inoculated overnight in liquid mCD at 28°C/180 rpm. The grown mycelium was filtered through miracloth and washed with 0.8 M of NaCl solution. The mycelium was digested in a Trichoderma harzianum lysing enzyme (Novozymes) suspension (150 mg in 15 ml 0.8 M of NaCl) for 1 h with soft shaking at 100 rpm at 30°C. Protoplast quality and quantity were checked via microscopy. Protoplasts were separated from cell fragments by filtering through two layers of Miracloth and precipitated at 2870 g for 5 min at 4°C. The pellet was resuspended in solution 1 (0.8 M of NaCl, 10 mM of $CaCl_2$ and 50mM of TrisHCl pH 7.5). Five microgram of plasmid DNA was added to 100μl of protoplast suspension and inoculated on ice for 5 min. One milliliter of solution 2 (40% PEG 4000, 0.8 M of NaCl, 50 mM of $CaCl_2$ and 50mM of TrisHCl pH 7.5) was added and the mixture was incubated at room temperature for 20 min. 15 ml CZDS soft-top agar (3.5% of Czapek Dox broth, 0.7% of agar and 1M of sorbitol) were added to the transformation mixture and poured onto Czapek-Dox (3% of sucrose, 0.3% of $NaNO_3$, 0.1% of KH2PO4, 0.05% of KCl, 0.05% of $MgSO_4$ 7 × H_2O, 0.001% of $FeSO_4$, × 7H_2O, 1.5% of agar, pH 7.3) plates supplemented with sorbitol (1M) and appropriate markers and incubated at 28°C until colonies were formed.

Deletion of different AOH-cluster genes

For the design of the deletion constructs two protospacer sequences per gene were chosen to produce two different sgRNAs from the respective constructs. The sequences were introduced into the sgRNA-constructs by PCR using pFC334 as a template to obtain two PCR-fragments per protospacer that were cloned into pFC332 (first protospacer) and pFC330 (second protospacer) respectively (Table 4).

Table 4. Plasmids used in this study.

Plasmid	Content	Reference
pFC332	*tef1*(p)::*cas9*::*tef1*(t); *gpdA*(p)::*gpdA*(t); *hph*; *ampR*; AMA1	Uffe H. Mortensen, Technical University of Denmark
pFC330	*tef1*(p)::*cas9*::*tef1*(t); *gpdA*(p)::*gpdA*(t); Af*pyrG*; *ampR*; AMA1	Uffe H. Mortensen, Technical University of Denmark
pFC334	*tef1*(p)::*cas9*::*tef1*(t); *gpdA*(p)::sgRNA-*AnyA*::*gpdA*(t); Af*pyr4*; *ampR*; AMA1	Uffe H. Mortensen, Technical University of Denmark
pTYGSarg2.0	*amyB*(p)::*amyB*(t); *adh1*(p)::*adh1*(t); *gpdA*(p)::*gpdA*(t); *enoA*(p)::*enoA*(t); *ampR*; *argB*; URA3	Colin M. Lazarus, University of Bristol
pTYGSade2.0	*amyB*(p)::*amyB*(t); *adh1*(p)::*adh1*(t); *gpdA*(p)::*gpdA*(t); *enoA*(p)::*enoA*(t); *ampR*; *adeA*; URA3	Colin M. Lazarus, University of Bristol
Efimov-gpdA(p)	*gpdA*(p)::*trpC*(t); *ampR; pyr4*	
pMW15	*amyB*(p)::*pksI*::*amyB*(t); *adh1*(p)::*adh1*(t); *gpdA*(p)::*gpdA*(t); *enoA*(p)::*enoA*(t); *ampR*; *argB*; URA3	This study
pMW28	*amyB*(p)::*pksI*::*amyB*(t); *adh1*(p)::*omtI*::*enoA*(t); *ampR*; *argB*; URA3	This study
pMW30	*amyB*(p)::*pksI*::*amyB*(t); *adh1*(p)::*omtI*::*adh1*(t); *gpdA*(p)::*moxI*::*gpdA*(t); *enoA*(p)::*sdrI*::*enoA*(t); *ampR*; *argB*; URA3	This study
pMW31	*amyB*(p)::*pksI*::*amyB*(t); *adh1*(p)::*omtI*::*adh1*(t); *gpdA*(p)::*moxI*::*enoA*(t); *ampR*; *argB*; URA3	This study
pMW32	*amyB*(p)::*pksI*::*amyB*(t); *adh1*(p)::*omtI*::*adh1*(t); *gpdA*(p)::*sdrI*::*enoA*(t); *ampR*; *argB*; URA3	This study
pMW36	*tef1*(p)::*cas9*::*tef1*(t); *gpdA*(p)::*pksI*-sgRNA-cassette1::*gpdA*(t); *hph*; *ampR*; AMA1	This study
pMW53	*tef1*(p)::*cas9*::*tef1*(t); *gpdA*(p):: *aohR*-sgRNA-cassette1::*gpdA*(t); *hph*; *ampR*; AMA1	This study
pMW55	*tef1*(p)::*cas9*::*tef1*(t); *gpdA*(p):: *aohR*-sgRNA-cassette2::*gpdA*(t); Af*pyrG*; *ampR*; AMA1	This study
pMW56	*tef1*(p)::*cas9*::*tef1*(t); *gpdA*(p):: *sdrI*-sgRNA-cassette1::*gpdA*(t); *hph*; *ampR*; AMA1	This study
pMW57	*tef1*(p)::*cas9*::*tef1*(t); *gpdA*(p):: *sdrI*-sgRNA-cassette2::*gpdA*(t); Af*pyrG*; *ampR*; AMA1	This study
pMW58	*gpdA*(p)::*aohR*::*trpC*(t); *ampR*; *pyr4*	This study
pMW61	*tef1*(p)::*cas9*::*tef1*(t); *gpdA*(p)::pksI-sgRNA-cassette2::*gpdA*(t); Af*pyrG*; *ampR*; AMA1	This study
pMW70	*tef1*(p)::*cas9*::*tef1*(t); *gpdA*(p):: *moxI*-sgRNA-cassette1::*gpdA*(t); *hph*; *ampR*; AMA1	This study
pMW71	*tef1*(p)::*cas9*::*tef1*(t); *gpdA*(p):: *moxI*-sgRNA-cassette2::*gpdA*(t); Af*pyrG*; *ampR*; AMA1	This study
pMW72	*amyB*(p)::*amyB*(t); *adh1*(p)::*doxI*::*enoA*(t); *ampR*; *adeA*; URA3	This study
pMW76	*tef1*(p)::*cas9*::*tef1*(t); *gpdA*(p):: *doxI*-sgRNA-cassette1::*gpdA*(t); *hph*; *ampR*; AMA1	This study
pMW77	*tef1*(p)::*cas9*::*tef1*(t); *gpdA*(p):: *doxI*-sgRNA-cassette2::*gpdA*(t); Af*pyrG*; *ampR*; AMA1	This study

For the PCR the oligonucleotides crispr fw 2.0 and crispr re 2.0 were paired with the oligonucleotides that contained the gene specific sequences '*gene*-6bp re 1 or 2' and '*gene*-Proto fw 1 or 2' (Table 3).

Overexpression of aohR

For the overexpression construct the *aohR*-ORF was amplified by PCR using the primers 'aohR oe fw/re' (Table 3) and *A. alternata* genomic DNA as template. The PCR fragment was cloned in the linearized vector Efimov-*gpdA*(p) (Table 4) via Gibson Assembly.

Heterologous expression of AOH-cluster genes

The ORFs of the AOH-cluster genes were amplified by PCR using the primers '*gene*-ory fw/re' (Table 3) and *A. alternata* genomic DNA as template. The PCR fragments were cloned in the restriction digested vectors pTYGSarg2.0 and pTYGSade2.0 (Table 4) via Gibson Assembly.

Metabolite extraction

For the extraction of secondary metabolites *A. alternata* and *A. oryzae* strains were grown for 7 days in their respective production medium either on 60 mm plates at 28°C or in 100 ml of liquid medium shaking at 180 rpm and 28°C. For extraction from solid medium three disks from each plate were excised with the back of a blue pipette tip and extracted by shaking with 1 ml of ethyl acetate for 1 h. For extraction from liquid culture the grown mycelium was filtered, washed with sterile H_2O and lyophilized overnight. The dried mycelium was extracted with 10ml of ethyl acetate per gram by shaking for 1 h at room temperature.

Thin layer chromatography analysis

For the qualitative analysis of the *A. alternata* and *A. oryzae* extracts, 15 µl of crude extract was put on TLC plates coated with 0.25 mm silica gel as stationary phase (Pre-coated TLC plates SIL G-25, Macherey-Nagel, Düren, Germany). The mobile phase for separation of the metabolites consisted of 50% of toluol, 40% of ethylacetate and 10% of formic acid. The mycotoxins were visualized using UV light at 254 nm.

HPLC analysis

Flexar LC system (Perkin Elmer, Rodgau, Germany), Separation: Brownlee Analytical column 150 × 4.6 mm, 5 µm particle size (Perkin Elmer, Rodgau, Germany). Eluent A was acetonitril and Eluent B was water with 0.1% of formic acid. A linear gradient with a flow rate of 0.5 ml min^{-1} was applied. The gradient was as follows: 0 min: 10% A,

0–20 min: 10% A → 100% A, 20–30 min isocratic with 100% A. The DAD recorded at 254 nm with a rate of 5 Hz.

LC-DAD-MS analysis of A. oryzae *samples*

Ethyl acetate extracts of the *A. oryzae* samples were analyzed by LC-DAD-MS. Therefore, the extracts were first vortexed and centrifuged. Then 100 µl of the supernatants were evaporated to dryness under a nitrogen stream. The residues were resolved in 100 µl acetonitrile:methanol mixture (1:1, v:v) and measured by a Triple TOF 5600 mass spectrometer (AB Sciex, Darmstadt, Germany) equipped with a 1290 Infinity LC system (Agilent, Waldbronn, Germany). The LC-DAD-MS system was controlled by the software Analyst TF 1.6.0. LC separation was done on an Acquity HSS T3 (2.1 × 100 mm, 1.8 µm particle size; Waters, Eschborn, Germany) equipped with a pre-column (Waters Acquity HSS T3, 2.1 × 5 mm, 1.8 µm particle size) and a pre-in-line filter (Krudkatcher, 0.5 µm; Phenomenex, Aschaffenburg, Germany). Eluent A was an aqueous 10 mM of ammonium formate buffer with 0.05% formic acid and eluent B was acetonitrile:methanol mixture (1:1, v:v). A linear gradient was used with a flow rate of 0.3 ml min^{-1} and the following elution profile: 0.0–2.0 min isocratic with 3% B, 2.0–53.0 min from 3%–95% B, 53.0–58.0 min isocratic with 95% B, 58.0–58.5 min from 95%–3% B, and 58.5–63.0 min isocratic with initial conditions. The column oven was set to 30 °C and the injection volume was 2 µl. The DAD recorded data from 220 to 600 nm with a sampling rate of 10 Hz. Samples were measured twice, in negative and in positive mode. The DuoSpray source was operated in ESI mode using the following source parameters: Curtain gas 45 psi, ion spray voltage −4500 V and +5500 V, respectively, ion source gas-1 60 psi, ion source gas-2 50 psi, ion source gas-2 temperature 650°C. The declustering potential was adjusted to −100 V and +100 V respectively. The MS full scans were recorded from m/z 100 to 1000 with an accumulation time of 100 ms and a collision energy voltage of −10 V and +10 V respectively. The MS/MS spectra (product ion) were recorded from m/z 50 to 1000 in the high sensitivity mode with an accumulation time of 40 ms, a collision energy voltage of −45 V and +45V, respectively, and a collision energy spread of 25 V. Nitrogen was used as collision gas. All used organic solvents and reagents were of LC grade quality and the water was taken from an in-house ultrapure water system (LaboStar; Siemens, Erlangen, Germany) at 0.055 µS cm^{-1}. Data analyses were done by the software PeakView 2.2.0, MasterView 1.1 and FormulaFinder 2.2.0.

Infection assays

The *A. alternata* ATCC 66981 wild-type strain able to produce AOH and the respective *pksI*-deficient strain not able to produce AOH have been used throughout the infection experiments. The strains were pregrown for 7 days at 25 °C on 92 mm petri-dishes (Greiner Biolabs) containing mCDB medium For inoculation of tomatoes, citrus or apple spores from 7-day old precultures have been suspended in Tween salt solution (1 g Tween 80 l^{-1}, 9 g NaCl l^{-1}) to reach a spore count of 10^6 spores ml^{-1}. Tomatoes have been surface disinfected with 70% of ethanol and carved by a sterile scalpel in a length of 1 cm. Afterwards, 10 µl of the respective spore suspension containing either the wild-type strain or the *pksI* deficient strain have been carefully applied into the wound using a sterile pipette tip. Apple and citrus fruits were surface sterilized by 2% of sodium hypohlorite and wounded with a cork borer 5 mm in diameter. Wounds were inoculated by mycelium plugs from the margin of actively growing colonies. In order to proof the hypothesis that AOH itself is involved in the infection process as virulence promoting metabolite, a solution of AOH dissolved in methanol (5 µg $µl^{-1}$) has been applied to tomatoes, citrus or apple infected with the AOH deficient *A. alternata* strain. Infected fruits have been incubated for 7 days at 28°C and the area of infection was measured two times orthogonal to each other. All experiments were done in biological triplicates.

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References

Babiceanu, M.C., Howard, B.A., Rumore, A.C., Kita, H. and Lawrence, C.B. (2013) Analysis of global gene expression changes in human bronchial epithelial cells exposed to spores of the allergenic fungus, *Alternaria alternata*. *Frontiers in Microbiology*, **4**, 196.

Bensassi, F., Gallerne, C., Sharaf El Dein, O., Hajlaoui, M.R., Bacha, H. and Lemaire, C. (2012) Cell death induced by the *Alternaria* mycotoxin alternariol. *Toxicology in Vitro*, **26**, 915–923.

Breitenbach, M. and Simon-Nobbe, B. (2002) The allergens of *Cladosporium herbarum* and *Alternaria alternata*. *Chemical Immunology*, **81**, 48–72.

Chaumpluk, P., Plubcharoensook, P. and Prasongsuk, S. (2016) Rapid detection of aflatoxigenic *Aspergillus* sp. in herbal specimens by a simple, bendable, paper-based lab-on-a-chip. *Biotechnology Journal*, **11**, 768–779.

Cho, Y. (2015) How the necrotrophic fungus *Alternaria brassicicola* kills plant cells remains an enigma. *Eukaryotic Cell*, **14**, 335–344.

Chooi, Y.H., Muria-Gonzalez, M.J., Mead, O.L. and Solomon, P.S. (2015) SnPKS19 encodes the polyketide synthase for alternariol mycotoxin biosynthesis in the wheat pathogen *Parastagonospora nodorum*. *Applied and Environment Microbiology*, **81**, 5309–5317.

Combina, M., Dalcero, A., Varsavsky, E., Torres, A., Etcheverry, M., Rodriguez, M., *et al.* (1999) Effect of heat treatments on stability of alternariol, alternariol monomethyl ether and tenuazonic acid in sunflower flour. *Mycotoxin Research*, **15**, 33–38. doi: 10.1007/BF02945212

Dang, H.X., Pryor, B., Peever, T. and Lawrence, C.B. (2015) The *Alternaria* genomes database: a comprehensive resource for a fungal genus comprised of saprophytes, plant pathogens, and allergenic species. *BMC Genomics*, **16**, 239.

Demuner, A.J., Barbosa, L.C., Miranda, A.C., Geraldo, G.C., da Silva, C.M., Giberti, S., *et al.* (2013) The fungal phytotoxin alternariol 9-methyl ether and some of its synthetic analogues inhibit the photosynthetic electron transport chain. *Journal of Natural Products*, **76**, 2234–2245.

Dong, Z.G., Liu, G.T., Dong, Z.M., Qian, Y.Z., An, Y.H., Miao, J.A. and Zhen, Y.Z. (1987) Induction of mutagenesis and transformation by the extract of *Alternaria alternata* isolated from grains in Linxian, China. *Carcinogenesis*, **8**, 989–991.

EFSA. (2011) Scientific opinion on the risks for animal and public health related to the presence of *Alternaria* toxins in feed and food. *EFSA Journal*, **9**, 2407.

Escrivá, L., O'ueslate, S., Font, G. and Manyes, L. (2017) *Alternaria* mycotoxins in food and feed. An overview. *Journal of Food Quality*, **2017**, 1569748.

Fehr, M., Pahlke, G., Fritz, J., Christensen, M.O., Boege, F., Altemöller, M., *et al.* (2009) Alternariol acts as a topoisomerasse poison, preferentially affecting the II alpha isoform. *Molecular Nutrition & Food Research*, **53**, 441–451.

Fleck, S.C. (2013) *In vitro-Studien zum genotoxischen Potential und zum Metabolismus von Mykotoxinen mit Resorcylsäurelakton und Perylenchinon-Struktur*. Karlruhe: KIT.

Furutani, Y., Bezerra, A.G.J., Waschuk, S., Sumii, M., Brown, L.S. and Kandori, H. (2004) FTIR spectroscopy of the K photointermediate of *Neurospora* rhodopsin: structural changes of the retinal, protein, and water molecules after photoisomerization. *Biochemistry*, **43**, 9636–9646.

Garganese, F., Schena, L., Siciliano, I., Prigigallo, M.I., Spadaro, D., De Grassi, A., *et al.* (2016) Characterization of citrus-associated *Alternaria* species in mediterranean areas. *PLoS ONE*, **11**, e0163255.

Graf, E., Schmidt-Heydt, M. and Geisen, R. (2012) HOG MAP kinase regulation of alternariol biosynthesis in *Alternaria alternata* is important for substrate colonization. *International Journal of Food Microbiology*, **157**, 353–359.

Hayes, T., Rumore, A., Howard, B., He, X., Luo, M., Wuenschmann, S., *et al.* (2018) Innate immunity induced by the major allergen alt a 1 from the fungus *Alternaria* is dependent upon toll-like receptors 2/4 in human lung epithelial cells. *Frontiers in Immunology*, **9**, 1507.

Jarolim, K., Del Favero, G., Pahlke, G., Dostal, V., Zimmermann, K., Heiss, E., *et al.* (2017) Activation of the Nrf2-ARE pathway by the *Alternaria alternata* mycotoxins altertoxin I and II. *Archives of Toxicology*, **91**, 203–216.

Johann, S., Rosa, L.H., Rosa, C.A., Perez, P., Cisalpino, P.S., Zani, C.L. and Cota, B.B. (2012) Antifungal activity of altenusin isolated from the endophytic fungus *Alternaria* sp. against the pathogenic fungus *Paracoccidioides brasiliensis*. *Revista Iberoamericana de Micología*, **29**, 205–209.

Lee, H.B., Patriarca, A. and Magan, N. (2015) Alternaria in food: ecophysiology, mycotoxin production and toxicology. *Mycobiology*, **43**, 93–106.

Lehmann, L., Wagner, J. and Metzler, M. (2006) Estrogenic and clastogenic potential of the mycotoxin alternariol in cultured mammalian cells. *Food and Chemical Toxicology*, **44**, 398–408.

Liu, G.T., Qian, Y.Z., Zhang, P., Dong, Z.M., Shi, Z.Y., Zhen, Y.Z., *et al.* (1991) Relationships between *Alternaria alternata* and oesophageal cancer. *IARC Scientific Publications*, (151), 258–262.

Meena, M., Gupta, S.K., Swapnil, P., Zehra, A., Dubey, M.K. and Upadhyay, R.S. (2017) *Alternaria* toxins: potential virulence factors and genes related to pathogenesis. *Frontiers in Microbiology*, **8**, 1451.

Pero, R.W., Posner, H., Blois, M., Harvan, D. and Spalding, J.W. (1973) Toxicity of metabolites produced by the "*Alternaria*". *Environmental Health Perspectives*, **4**, 87–94.

Pinto, V.E. and Patriarca, A. (2017) *Alternaria* species and their associated mycotoxins. *Methods in Molecular Biology*, **1542**, 13–32.

Pollock, G.A., DiSabatino, C.E., Heimsch, R.C. and Hilbelink, D.R. (1982) The subchronic toxicity and teratogenicity of alternariol monomethyl ether produced by *Alternaria solani*. *Food and Chemical Toxicology*, **20**, 899–902.

Rychlik, M., Zappa, G., Anorga, L., Belc, N., Castanheira, I., Donard, O.F.X., *et al.* (2018) Ensuring food integrity by metrology and FAIR data principles. *Frontiers in Chemistry*, **6**, 49.

Saha, D., Fetzner, R., Burkhardt, B., Podlech, J., Metzler, M., Dang, H., *et al.* (2012) Identification of a polyketide synthase required for alternariol (AOH) and alternariol-9-methyl ether (AME) formation in *Alternaria alternata*. *PLoS ONE*, **7**, e40564.

Scharf, D.H., Heinekamp, T. and Brakhage, A.A. (2014) Human and plant fungal pathogens: the role of secondary metabolites. *PLoS Path*, **10**, e1003859.

Schrader, T.J., Cherry, W., Soper, K., Langlois, I. and Vijay, H.M. (2001) Examination of *Alternaria alternata* mutagenicity and effects of nitrosylation using the Ames Salmonella test. *Teratogenesis, Carcinogenesis, and Mutagenesis*, **21**, 261–274.

Schulz, M.C., Schumann, L., Rottkord, U., Humpf, H.U., Gekle, M. and Schwerdt, G. (2018) Synergistic action of the nephrotoxic mycotoxins ochratoxin A and citrinin at nanomolar concentrations in human proximal tubule-derived cells. *Toxicology Letters*, **291**, 149–157.

Speijers, G.J. and Speijers, M.H. (2004) Combined toxic effects of mycotoxins. *Toxicology Letters*, **153**, 91–98.

Stoll, D.A., Link, S., Kulling, S., Geisen, R. and Schmidt-Heydt, M. (2014) Comparative proteome analysis of *Penicillium verrucosum* grown under light of short wavelength shows an induction of stress-related proteins associated with modified mycotoxin biosynthesis. *International Journal of Food Microbiology*, **175**, 20–29.

Sun, J., Awakawa, T., Noguchi, H. and Abe, I. (2012) Induced production of mycotoxins in an endophytic fungus from the medicinal plant *Datura stramonium* L. *Bioorganic & medicinal chemistry letters*, **22**, 6397–6400.

Tiessen, C., Ellmer, D., Mikula, H., Pahlke, G., Warth, B., Gehrke, H., *et al.* (2017) Impact of phase I metabolism on uptake, oxidative stress and genotoxicity of the emerging mycotoxin alternariol and its monomethyl ether in esophageal cells. *Archives of Toxicology*, **91**, 1213–1226.

Touhami, N., Soukup, S.T., Schmidt-Heydt, M., Kulling, S.E. and Geisen, R. (2018) Citrinin as an accessory establishment factor of *P. expansum* for the colonization of apples. *International Journal of Food Microbiology*, **266**, 224–233.

Tsuge, T., Harimoto, Y., Akimitsu, K., Ohtani, K., Kodama, M., Akagi, Y., *et al.* (2013) Host-selective toxins produced by the plant pathogenic fungus *Alternaria alternata*. *FEMS Microbiology Reviews*, **37**, 44–66.

Wasil, Z., Pahirulzman, K.A.K., Butts, C., Simpson, T.J., Lazarus, C.M. and Cox, R.J. (2013) One pathway, many compounds: heterologous expression of a fungal biosynthetic pathway reveals its intrinsic potential for diversity. *Chemical Science*, **4**, 3845–3856.

Wenderoth, M., Pinecker, C., Voß, B. and Fischer, R. (2017) Establishment of CRISPR/Cas9 in *Alternaria alternata*. *Fungal Genetics and Biology*, **101**, 55–60.

Wojciechowska, E., Weinert, C.H., Egert, B., Trierweiler, B., Schmidt-Heydt, M., Horneburg, B., *et al.* (2014) Chlorogenic acid, a metaoblite identified by untargeted metabolome analysis in resistant tomatoes, inhibits the colonization by *Alternatria alternata* by inhibiting alternariol biosynthesis. *European Journal of Plant Pathology*, **139**, 735–747.

Zhen, Y.Z., Xu, Y.M., Liu, G.T., Miao, J., Xing, Y.D., Zheng, Q.L., *et al.* (1991) Mutagenicity of *Alternaria alternata* and *Penicillium cyclopium* isolated from grains in an area of high incidence of oesophageal cancer–Linxian, China. *IARC Scientific Publications*, (105), 253–257.