A Small Horizontally Transferred Gene Cluster Contributes to the Sporulation of *Alternaria alternata*

Mingshuang Wang [1,†], Huilan Fu[2,†], and Ruoxin Ruan[2,3,*]

[1]College of Life and Environmental Sciences, Hangzhou Normal University, Hangzhou, China

[2]Institute of Biotechnology, Zhejiang University, Hangzhou, China

[3]Hangzhou Academy of Agricultural Sciences, Hangzhou, China

[†]These authors contributed equally to this work.

*Corresponding author: E-mail: buffalo126@126.com.

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Abstract

Horizontal gene transfer (HGT) has been identified as an important source of genomic innovation in fungi. However, how HGT drove the evolution of *Alternaria alternata*, a necrotrophic fungus which can be ubiquitously isolated from soil and various plants and decaying plant materials is largely known. In this study, we identified 12 protein-encoding genes that are likely acquired from lineages outside Pezizomycotina. Phylogenetic trees and approximately unbiased comparative topology tests strongly supported the evolutionary origin of these genes. According to their predicted functions, these HGT candidates are involved in nitrogen and carbohydrate metabolism. Especially, five genes of them were likely transferred as a physically linked cluster from Tremellales (Basidiomycota). Functionally knocking out the five-gene cluster in an *A. alternata* isolate causing citrus brown spot resulted in an 80% decrease in asexual spore production in the deletion mutant. We further knocked out each of these five genes in this cluster and the resultant single-gene deletion mutants exhibited a various degree of reduction in spore production. Except for conidiation, functions of these genes associated with vegetative growth, stress tolerance, and virulence are very limited. Our results provide new evidence that HGT has played important roles over the course of the evolution of filamentous fungi.

Key words: *Alternaria alternata*, horizontal gene transfer, genomic innovation, gene cluster, spore production.

Introduction

Horizontal gene transfer (HGT) is the mobilization and integration of genetic material between reproductively isolated species which contrasts the vertical inheritance of genes passed from parent to progeny (Fitzpatrick 2012; Soanes and Richards 2014). HGT is ubiquitous among prokaryotes and is considered to profoundly shape the evolution of bacteria (Koonin et al. 2001). In eukaryotes, although HGT appears to happen less frequently as compared with bacteria, it is also considered to be an important force driving genomic innovation, especially in unicellular organisms (Keeling and Palmer 2008; Fitzpatrick 2012). HGT genes in fungal lineages may serve various functions such as secondary metabolites biosynthesis, nutrients utilization, host infection, and adaptation to harsh environments (Gojković et al. 2004; Hall et al. 2005; Friesen et al. 2006; Novo et al. 2009; Richards, Leonard, et al. 2011; Soanes and Richards 2014). One of the best-characterized examples is a gene encoding a critical virulence factor ToxA which was transferred from the wheat blotch pathogen *Stagonospora nodorum* to the wheat tan spot pathogen *Pyrenophora tritici-repentis*, leading to the emergence of a new damaging disease of wheat (Friesen et al. 2006). A recent study demonstrated that the horizontal transfer of *ToxA* between these species was likely facilitated by a type II DNA transposon. This horizontal transfer event is now in the process of extensive decay due to the repeated insertion of new transposons and subsequent rounds of targeted mutation (McDonald et al. 2019).

In recent years, with the widely available fungal genome data, an increasing number of HGT events that happened in fungi were discovered, showing that HGT in fungi occurs more frequently than previously thought (Kurland et al. 2003; Soanes and Richards 2014). For example, 11 genes were identified to be transferred from bacteria into the common ancestors of the genus *Colletotrichum* (Jaramillo et al. 2015). A recent study showed that >90 genes were likely

transferred between *Colletotrichum* and Magnaporthales, providing a new perspective on the scale of HGT between fungi (Qiu et al. 2016). Functions of those HGT genes are predicted and linked to some certain metabolic processes like lipid and sugar metabolism and degrading plant cell wall, however, experimental evidence confirming a phenotype for most HGT genes are yet to be discovered.

The tangerine pathotype of the necrotrophic fungus *Alternaria alternata* is the causal agent of citrus brown spot, which can result in significant losses of both yield and marketability for tangerines worldwide (Peever et al. 2002). It can produce a unique host-selective toxin (ACT toxin) that kills host cells prior to invading the host. The ability to produce the ACT toxin is critically required for *A. alternata* pathogenesis (Tsuge et al. 2013). Previously, the gene cluster responsible for the biosynthesis of the ACT toxin was identified to be composed of 25 genes (Wang et al. 2016). Interestingly, the phylogenetic analysis suggested that 4 of the 25 genes were likely transferred from distantly related fungi and functional experiments demonstrated that three of them are essential for the virulence of *A. alternata* (Tanaka and Tsuge 2000; Wang et al. 2019). These results led us to explore whether other HGT events happened in this species, as well as their biological functions. In this study, we performed a genome-wide analysis of *A. alternata* and identified 12 genes that are likely acquired from lineages outside Pezizomycotina. We further focused on a five-gene cluster that was likely transferred from distantly related Tremellales (Basidiomycota) to the tangerine pathotype of *A. alternata*, we also carried out a series experiments to reveal their potential functions.

Results and Discussion

To examine whether any of the *A. alternata* Z7 genes originated via HGT, we calculated the Alien Index of all genes (Gladyshev et al. 2008; Wisecaver et al. 2016). A total of 188 genes show AI >0 and at least 80% of their top 200 BLASTp hits with a taxonomic classification other than Dothideomycetes. The validity of these 188 HGT candidates was further examined phylogenetically. The phylogenetic trees for most of these 188 HGT candidates were weakly supported, but the evolutionary origin of 12 of these genes was strongly supported to be outside Pezizomycotina (table 1 and supplementary figs. S1–S12, Supplementary Material online). The approximately unbiased (AU) test for each of the 12 genes significantly rejected the hypothesis that they formed a monophyletic group with the rest of the sequences from Pezizomycotina (table 1). As the genes inferred to have undergone HGT are also found in other *Alternaria* species, we infer that the HGT events occurred before the divergence of the Z7 strain from the other *Alternaria* genomes examined and not after (fig. 1). Specifically, ten genes are only found in the genomes of species in *Alternaria* Clade I, the remaining two of the horizontally transferred genes are found in the genomes of species in *Alternaria* Clades I and II (fig. 1). Of these HGT genes, seven are likely acquired from bacteria. None of these seven HGT candidates of bacterial origin has introns consistent with their prokaryotic origin (table 1).

According to their predicted functions, these HGT genes include nitrogen metabolite repression regulator NmrA-like protein, epimerase, dehydrogenase, hexose transporter, neuraminidase, oxidoreductase, and carboxylesterase (table 1). Previously, the horizontal transfer of NmrA-like protein and hexose transporter have been recorded to occur between oomycetes and fungi (Richards, Soanes, et al. 2011; Soanes and Richards 2014). Notably, three bacteria origin genes (*AALT_g384*, *AALT_g7038*, and *AALT_g9829*) contain the NmrA-like family domain. The NmrA was a repressor of the transcription factor AreA, which regulates the expression of nitrogen-catabolic permeases and enzymes under nitrogen starvation (Wong et al. 2007). Thus, we speculated that these HGT genes might participate in nitrogen metabolism and utilization in *A. alternata*.

There are five HGT genes that are physically clustered genes and they almost always appear on the gene phylogeny as sisters to sequences from *Cryptococcus* (Basidiomycota), *Ilyonectria* (Sordariomycetes), or *Penicillium* (Eurotiomycetes) fungi (fig. 2 and supplementary figs. S7–S11, Supplementary Material online). Phylogenetic gene trees of *AALT_g11771* and *AALT_g11773* are nearly composed entirely of bacterial genes (fig. 2*C* and *E*), therefore the HGT of these two genes should have occurred earlier from bacteria to a small number of fungi. The phylogeny of gene *AALT_g11769*, *AALT_g11770*, *AALT_g11771*, and *AALT_g11772* showed that *A. alternata* was placed within a clade that contains multiple Basidiomycota species (mostly Tremellales, fig. 2*A–D*), so the direction of transfer was probably from *Tremellales* to some specific Pezizomycotina species. We observed that *Ilyonectria europaea* has a complete cluster while both *Penicillium flavigenum* and *Cryptococcus gattii* have four clustered genes that are highly similar with four of these five genes (fig. 3); *Penicillium flavigenum* lacks the neuraminidase encoding gene homolog (*AALT_g11771*) while *Cryptococcus gattii* lacks the oxidoreductase encoding gene homolog (*AALT_g11772*) (fig. 3). However, the gene order and orientation are quite different among the clusters of these three genomes (fig. 3), so these genes were rearranged over the course of the evolution. A TBlastN search found that 68 out of 373 amino acid sequences of AALT_g11772 can find a good BLAST hit (75% identity) in the intergenic region between *ADV23719.1* and *ADV23793.1*, indicating that the orthologous gene in this species might have been subject to death (fig. 3). In addition, we also observed that the *Cryptococcus neoformans* var. grubii strain c45 contains all these five genes; however, these genes are located in three different genomic contigs, so we are not able to determine whether they are

Table 1

Summary List of the Five Horizontal Gene Transfers (HGTs) in *Alternaria alternata* Z7

Gene ID	Protein Length	Best BLAST Hit	Protein Identity	Donor Group	Number of Introns	Description	AU Test *P* Value
AALT_g384	303	KFE72029.1 Hyalangium minutum	62	Proteobacteria	0	NmrA-like family	—
AALT_g7038	291	WP_087098190.1 Nocardiopsis sp. JB363	59	Actinobacteria	0	NmrA-like family	—
AALT_g8642	306	WP_066531526.1-1855519-Sphingobium sp. EP60837	56	Proteobacteria	0	NAD dependent epimerase	—
AALT_g9829	319	WP_067140815.1 Mycobacterium sp. 1245852_3	54	Actinobacteria	0	NmrA-like family	—
AALT_g10982	143	WP_013568113.1 Terriglobus saanensis SP1PR4	64	Acidobacteria	0	hypothetical protein	—
AALT_g11232	330	XP_019043752.1 Kwoniella bestiolae	61	Basidiomycota	5	NAD dependent epimerase	1.00E-06
AALT_g11769	320	OWZ32807.1 Cryptococcus neoformans var_grubii_c45	77	Basidiomycota	5	short chain dehydrogenase	1.00E-04
AALT_g11770	554	KIR44027.1 Cryptococcus gattii_CA1280	79	Basidiomycota	5	hexose transporter	3.00E-88
AALT_g11771	396	KIR44012.1 Cryptococcus gattii_CA1280	84	Basidiomycota/ Bacteria	4	neuraminidase	—
AALT_g11772	377	OWZ30204.1 Cryptococcus neoformans_var_grubii_c45	75	Basidiomycota	4	NAD(P)-binding oxidoreductase	9.00E-07
AALT_g11773	332	OWZ30203.1 Cryptococcus neoformans_var_grubii_c45	81	Basidiomycota/ Proteobacteria	0	gfo/Idh/MocA family oxidoreductase	—
AALT_g12037	475	WP_108439341.1 Glaciimonas sp. PCH181	77	Proteobacteria	0	carboxylesterase	—

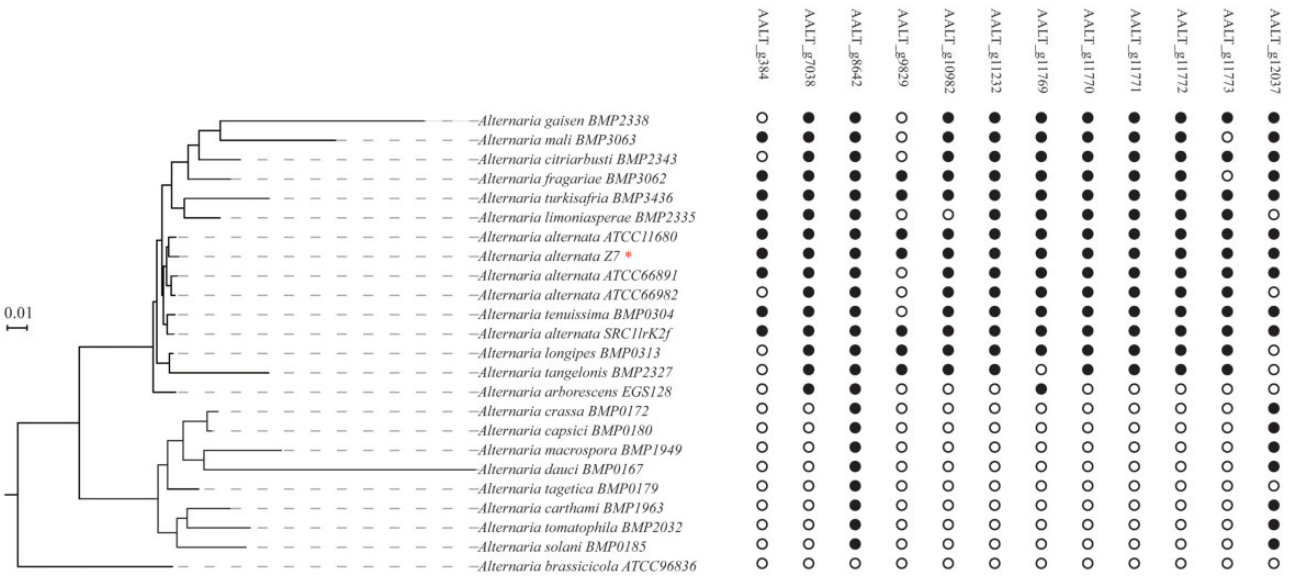

Fig. 1.—Distribution of the HGT gene orthologs within *Alternaria*.

physically linked with each other. From these results, the most likely scenario is that *AALT_g11771* and *AALT_g11773* are originally from bacteria; this cluster arose within Tremellales by duplication or HGT and then horizontally transferred to some specific Pezizomycotina species; subsequently, HGT may have happened among at least three phylogenetically disjunct Pezizomycotina classes to further distribute these genes, but it is uncertain what the direction is.

We then functionally annotated these five genes. All these genes encode enzymes except for *AALT_g11770*, which is a

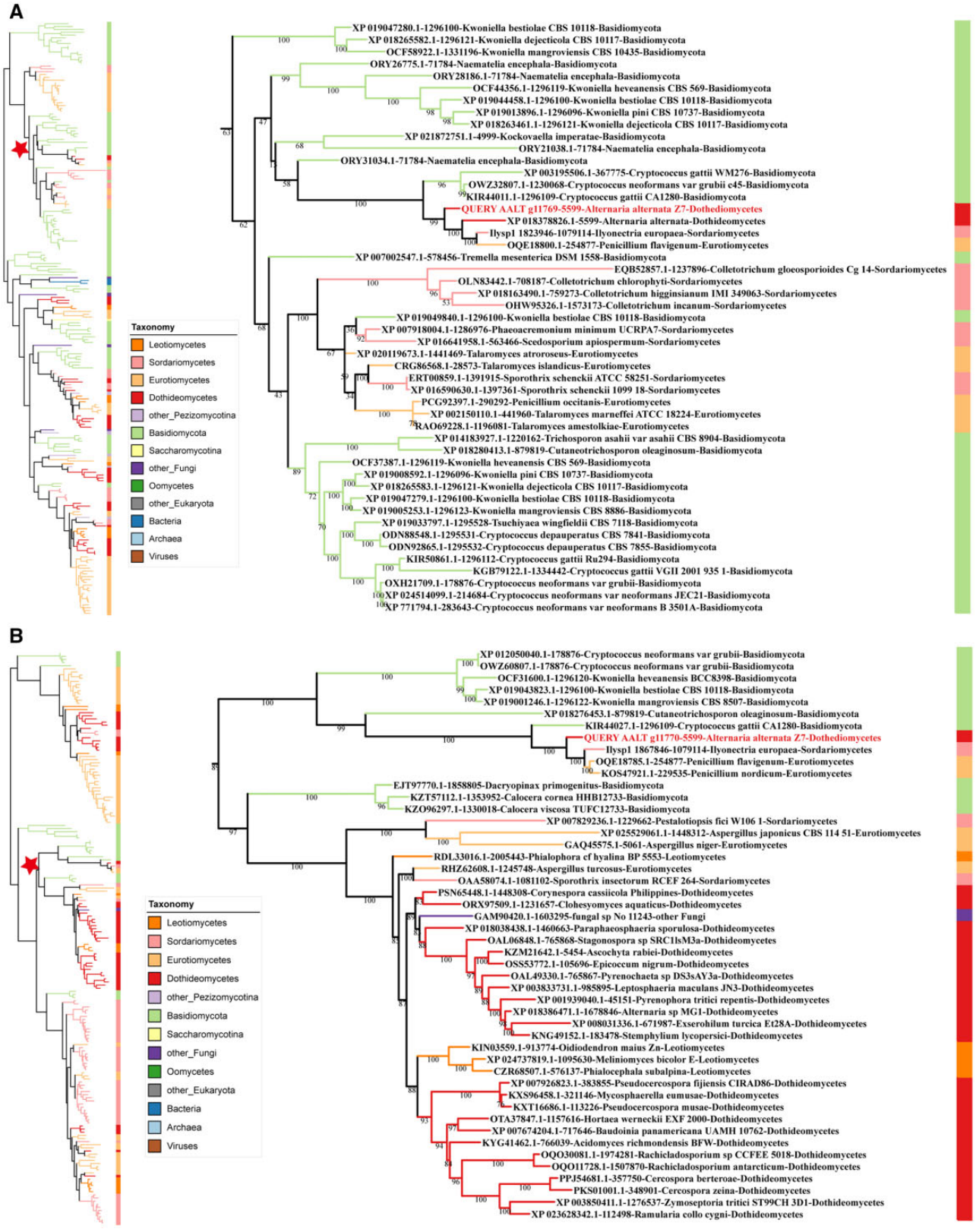

Fig. 2.—Horizontal transfer of a cluster of five genes in the tangerine pathotype of *Alternaria alternata* Z7. Phylogenetic evidence of the HGT of (*A*) *AALT_g11769*, (*B*) *AALT_g11770*, (*C*) *AALT_g11771*, (*D*) *AALT_g11772*, and (*E*) *AALT_g11773*. For each figure, the simplified full phylogeny is shown on the left while enlarged trees on the right. Red stars indicate where the clade is amplified. Branch colors indicate the taxonomic lineages to which the different taxa included in each phylogeny belong. The full phylogenetic trees with detailed information of the individual genes can be found in supplementary figures S7–S11, Supplementary Material online.

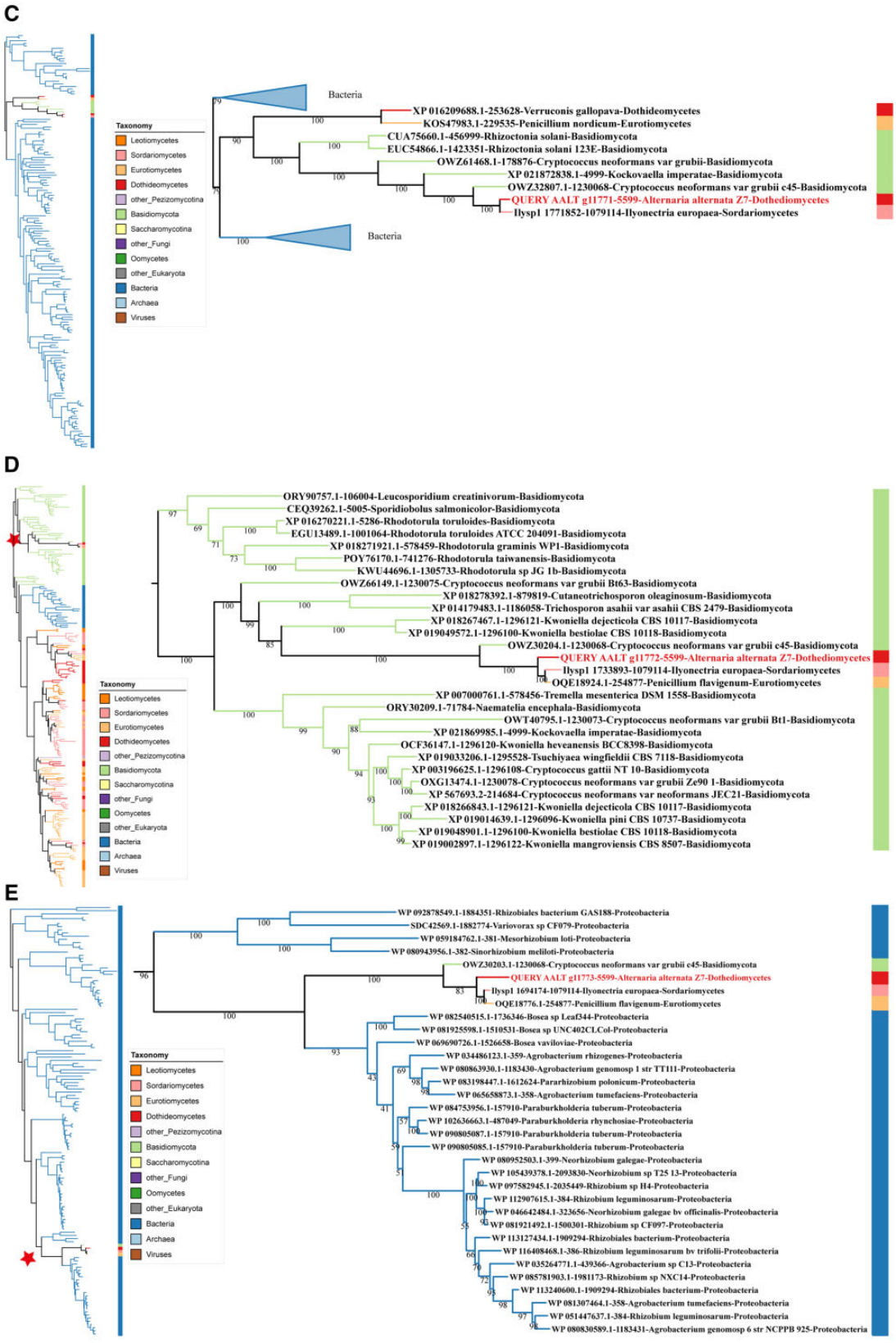

Fig. 2.—Continued.

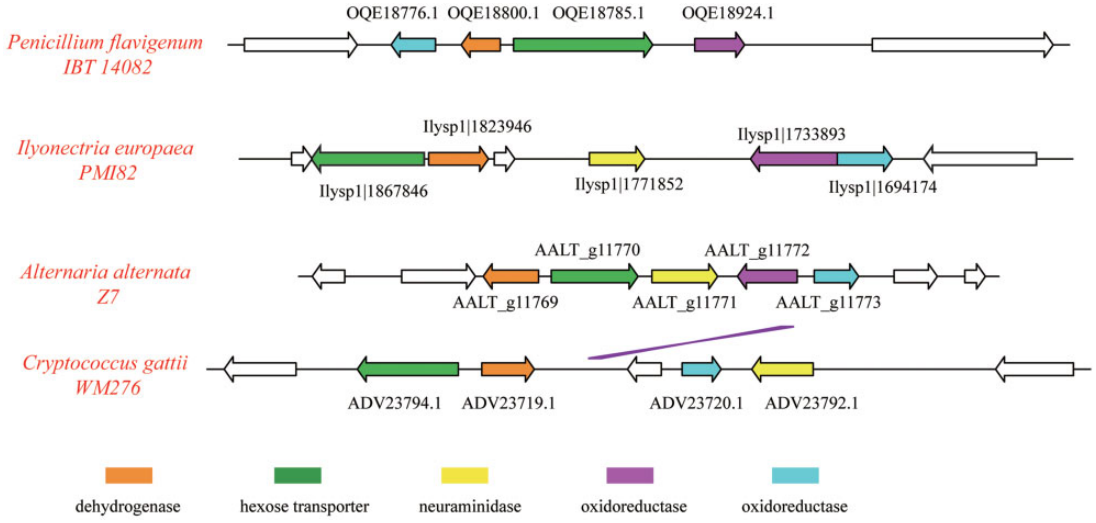

Fig. 3.—Conservation of synteny among the cluster of five genes in *Alternaria alternata* and the evolutionarily related clusters present in *Penicillium flavigenum*, *Ilyonectria europaea*, and *Cryptococcus gattii*. Orthologs among different species are marked with the same color. Arrows indicate gene direction.

hexose transporter (table 1). According to their predicted functions, these HGT candidates are involved in two biological processes: oxidation–reduction and carbohydrate metabolism (table 1). To further characterize this five-gene cluster, we constructed a deletion mutant lacking the entire cluster and examined its phenotypic characteristics upon osmotic, oxidative, fungicide, and cell wall stresses, as well as its asexual development and pathogenicity. We found that the mycelial growth of the mutant in potato dextrose agar (PDA) was similar when compared with that of the wild type (fig. 4*A*). However, the mutant formed a fluffy colony during asexual development and conidial production was decreased by 79.5% relative to wild-type conidial production (fig. 4). No other phenotypic differences between the mutant and the wild-type strains were found (supplementary fig. S13, Supplementary Material online). To confirm that the changed spore production of the mutant was truly caused by the deletion of the HGT cluster, we tried to make the complementation of the cluster to the mutant strains. However, the complementary strain was never obtained probably because the length of this cluster is too long. Alternatively, we knocked out each of these five genes in this cluster and complemented the *AALT_g11773* mutant with the wild-type *AALT_g11773* gene (supplementary figs. S14 and S15, Supplementary Material online). The results showed that those single-gene deletion mutants exhibited a various degree of reduction in spore production, and the defects in conidial sporulation of the *AALT_g11773* mutant can be mostly restored by the introduction of the wild-type *AALT_g11773* gene (fig. 4). These results indicate that this horizontally transferred gene cluster is critical for the conidiation of *A. alternata*.

In fungal genomes, the genes involved in metabolic pathways are often physically linked on chromosomes forming gene clusters. Horizontal transfers of gene clusters have been described in many fungal species(Slot and Rokas 2011; Campbell et al. 2012; Cheeseman et al. 2014; Marcet-Houben and Gabaldón 2016). A recent study found that most of the key enzymes needed to synthesize the ergot alkaloids and lolines in Clavicipitaceae were likely horizontally transferred from Eurotiomycetes (Marcet-Houben and Gabaldón 2016). These HGT events were speculated to have played a vital role in the capability of Clavicipitaceae to produce two key secondary metabolites to protect the plant hosts against herbivores, therefore favoring their interactions (Marcet-Houben and Gabaldón 2016). Previously, the 23-gene secondary metabolic gene cluster involved in the biosynthesis of the mycotoxin sterigmatocystin was shown to have been horizontally transferred from *Aspergillus* to *Podospora* (Slot and Rokas 2011). HGT of intact gene clusters would not only contribute to fungal metabolic diversity but also potentially provide its recipient with a competitive advantage offered by the ability to synthesize a novel secondary metabolite; for example, an independent study showed that *Podospora* produces sterigmatocystin (Matasyoh et al. 2011). Additionally, as one important driving force, HGT may also contribute to the genetic diversity of metabolic gene clusters, generating accessory functions (Slot 2017; Slot and Gluck-Thaler 2019). Recently, a systematic survey of the pan-genomes of four model fungal species implied that HGT is one important source in fungal pan-genome evolution, though it may occur at far lower rates (McCarthy and Fitzpatrick 2019). Although the horizontally transferred gene cluster in this study contains fewer genes, the five-gene cluster was shown to be critical for conidial production. These

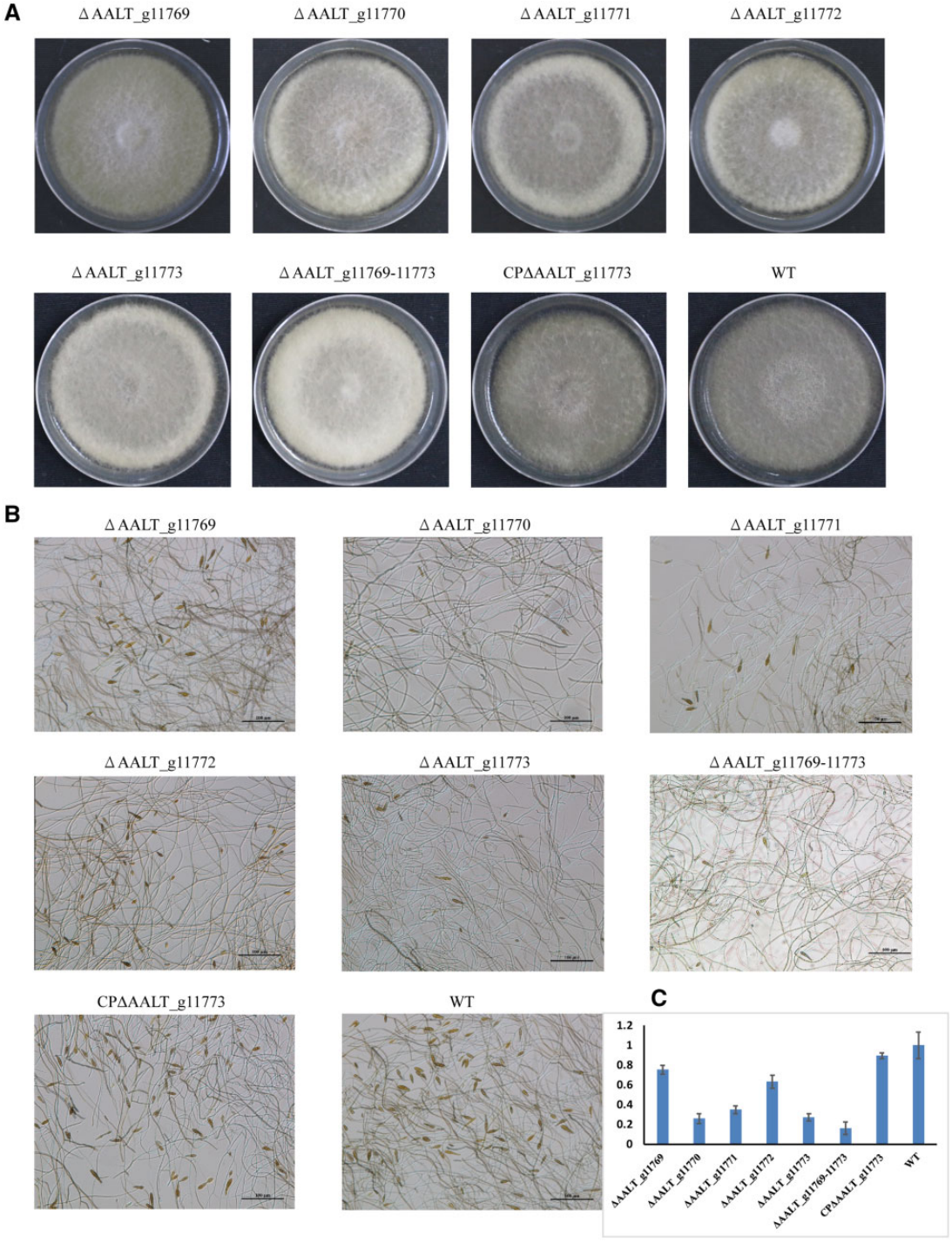

Fig. 4.—Conidial production in the wild type (WT) and in the HGT gene deletion/complementation mutants. (*A*) Vegetative growth of the wild-type and HGT gene deletion/complementation mutant strains on PDA at 25 °C for 5 days. (*B*) Light microscopy images of the formation of conidia by *Alternaria alternata* strains on PDA. (*C*) Relative conidia production in mutant strains. The conidia number of the wild type is arbitrarily set to 1.

results are in accordance with the opinion that HGT events, including ones involving the transfer of entire clusters, have played important roles in shaping the evolutionary innovation of filamentous fungi (Fitzpatrick 2012; Soanes and Richards 2014; Wisecaver and Rokas 2015; Wang et al. 2019).

Materials and Methods

Identification of CDC Genes That Underwent HGT

The assembled *A. alternata* Z7 genome and proteome were downloaded from GenBank under the accession number LPVP00000000 (Wang et al. 2016). To detect gene candidates that experienced HGT in *A. alternata* Z7, a phylogenomic pipeline was used with slightly modified (Shen et al. 2018). Briefly, we first performed a BLASTp search of the local NCBI's nonredundant protein database (nr, last access on October 25, 2018) using Z7 proteins as queries and then selected proteins with the following characteristics as HGT candidates for further phylogenetic analyses as described previously (Wisecaver et al. 2016; Shen et al. 2018): 1) an Alien Index (AI) score >0, 2) at least 80% of the top 200 BLASTp hits of the query protein are from organisms other than Dothideomycetes, and 3) the sequence identity of the query protein across its entire length to its best BLASTp hit is ≥50%.

All genes that fit these three criteria were used as query sequences in BLASTp searches against the nr database and phylogenetic trees of their most closely related sequences across the tree of life were constructed. The *Ilyonectria europaea* genome was also included in the analysis as it has all genes of the horizontally transferred gene cluster (Liao et al. 2019). To reduce the number of sequences used to build each phylogenetic tree, we used the top 300 hits from the BLAST results. The resulting homologs were first collapsed with CD-HIT v4.8.1 using a sequence identity threshold of 0.95 (Fu et al. 2012) and then aligned with MAFFT v7.023b using the E-INS-I strategy (Yamada et al. 2016) and trimmed with trimAl v1.4.rev11 using its automated1 strategy (Capella-Gutierrez et al. 2009). The maximum-likelihood (ML) phylogenetic trees were inferred using IQ-TREE 1.5.4, with the best model selected by ModelFinder (Nguyen et al. 2015; Kalyaanamoorthy et al. 2017) and with 1,000 bootstrap replicates.

For those HGT candidates, we used the Consel software, version V0.1i (Shimodaira and Hasegawa 2001; Shimodaira 2002) to perform the approximately unbiased (AU) comparative topology test between the unconstrained ML tree and the constrained ML tree in which the *Alternaria* gene sequence was forced to be monophyletic with the rest of the sequences from Dothideomycetes (Shimodaira 2002). All phylogenetic trees were visualized using ITOL version 3.0 (Letunic and Bork 2016).

Functional Analyses of Horizontally Transferred Genes

The reference *A. alternata* strain, Z7, was isolated from an infected citrus fruit from Zhejiang, China (Huang et al. 2015; Wang et al. 2016). Z7 and its derived mutants were stored in 20% glycerol solutions at −80 °C until use. The five-gene cluster and each HGT gene were knocked out using a fungal protoplast transformation protocol, as described previously (Wang et al. 2018). Briefly, the two flanking 600–1,000 bp fragments and a bacterial phosphotransferase B gene (HYG) were fused together and the resulting fragment was then introduced into fungal protoplasts using polyethylene glycol and $CaCl_2$. The transformants growing on a medium supplemented with 150 μg/ml hygromycin were selected and examined by PCR with specific primer pairs (supplementary fig. S7, Supplementary Material online). For genetic complementation, a full-length *AALT_g11773* fragment with its endogenous promoter was amplified by PCR and was inserted into the pA1300-NEO plasmid carrying a neomycin-resistance gene (Wang et al. 2015). The resultant plasmid was then transformed into protoplasts of a ΔAALT_g11773 mutant. Transformants were recovered from medium supplemented with neomycin (100 mg/l) and examined by PCR (supplementary fig. S7, Supplementary Material online). All the primers used in this study are listed in supplementary table S1, Supplementary Material online.

Wild-type and mutant strains were grown on regular solid potato dextrose agar (PDA) at 25 °C and asexual spore (conidial) production was inspected under a Nikon Eclipse 80i light microscope (Nikon, Tokyo, Japan) after incubating for 5 days (The sealing films on the petri dish were torn off in the last 2 days to promote spore formation). Conidia were collected with a 10 ml sterile solution of 0.05% (v/v) Tween 20 and filtered through lens wiping paper. Conidial concentration was quantified using a hemocytometer. To examine stress tolerance, mutant and wild-type strains were grown on PDA plates supplemented with either 1.5 mM CuSO4, 0.01% SDS, 250 μg/ml Congo red, 250 mM $CaCl_2$, 1 M NaCl, 25 μg/ml Azoxystrobin, 20 mM H_2O_2, or 2 mM tert-Butyl hydroperoxide. Each plate was inoculated with a 5-mm mycelial plug taken from the edge of a 5-day-old colony. The diameters of the colonies were measured after the plates were incubated at 25 °C for 7 days. Fungal virulence was assessed on *Citrus clementina* leaves inoculated by placing a 5 mm plug taken from the media for 2 days. Each strain was tested on at least five leaves and experiments were repeated two times.

Supplementary Material

Supplementary data are available at *Genome Biology and Evolution* online.

Acknowledgments

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