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LaeA and VeA are involved in growth morphology, asexual development, and mycotoxin production in *Alternaria alternata*

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ABSTRACT

Alternaria alternata is a common filamentous fungus that contaminates various fruits, grains and vegetables causing important economic losses to farmers and the food industry. *A. alternata* is a mycotoxigenic mould, which may jeopardize human and animal health. Two of the most common *A. alternata* mycotoxins found in food and feed are alternariol and alternariol monomethyl ether. In this study we examined the role of LaeA and VeA, two regulatory proteins belonging to the velvet family, which have been described to be involved in several functions in many fungi including secondary metabolism. We found that deletion of *laeA* and *veA* genes, respectively, greatly reduced sporulation and strongly compromised mycotoxin production, both *in vitro* or during pathogenesis of tomato fruits. We have also studied how the loss of *laeA* and *veA* may affect expression of genes related to alternariol and alternariol monomethyl ether biosynthesis (*pksJ* and *altR*), and to melanin biosynthesis (*cmrA*, *pksA*).

1. Introduction

The *Alternaria* genus of ascomycete fungi comprises saprophytic and pathogenic species causing plant diseases in many field crops and postharvest decay of various fruits, grains and vegetables. Besides their importance due to economic losses worldwide for farmers and the food industry, *Alternaria* is also a matter of concern due to its ability to produce a wide variety of different toxic secondary metabolites (mycotoxins). Among all *Alternaria* spp., *A. alternata* has been regarded as the most important mycotoxin-producing species (Barkai-Golan and Paster, 2008; EFSA, 2011). Although it is well documented that *Alternaria* mycotoxins are harmful for human and animals (Brugger et al., 2006; Liu et al., 1992; Pero et al., 1973; Pfeiffer et al., 2007; Pollock et al., 1982), currently there are no regulations on *Alternaria* toxins in food and feed in Europe or other regions of the world.

Since the discovery of aflatoxins produced by *Aspergillus* spp., many studies have focused on understanding the molecular mechanisms leading to mycotoxin biosynthesis. Although there have been significant advances in knowledge of the molecular regulation of some mycotoxins, still there is scarce information on the biosynthesis of *Alternaria* mycotoxins. Recently, Saha et al. (2012) have identified ten putative polyketide synthases (PKSs) in *A. alternata*, suggesting that one, PksJ, was supposedly responsible for catalyzing the first steps of the biosynthesis of alternariol (AOH) and alternariol monomethyl ether (AME), two of the most common *Alternaria* mycotoxins. Interestingly, they also found another gene, *altR*, that had homology to other fungal transcription factors, which was found to be involved in *pksJ* induction (Saha et al., 2012). A recently published report (Chooi et al., 2015) dealt with AOH and AME biosynthesis in the wheat pathogen *Parastagonospora nodorum*, which has been described to also produce AOH (Tan et al., 2009). In this latest study, a gene with close homology to *pksJ* was not found in the *P. nodorum* genome but, interestingly, it was reported that another protein, SnPKS19, was required for AOH biosynthesis and, additionally, SnPKS19 shared significant homology to PksI, a PKS that was also described by Saha et al. (2012) in the *A. alternata* genome. Hence, further investigation is required to verify the gene truly responsible of AOH synthesis in *A. alternata*.

The fungus *Aspergillus nidulans* is one of the best genetically characterized eukaryotic systems and has been quite useful for studying secondary metabolite biosynthesis mechanisms. For example, the heterotrimeric velvet complex was first characterized in this species (Bayram et al., 2008a). The velvet family proteins, LaeA, VeA and VelB, are fungal specific and have a marked functional plasticity in different species, but they are structurally highly conserved among ascomycetes and basidiomycetes (Calvo, 2008; Ni and Yu, 2007). In several fungal species the velvet complex has been found to be involved in the regulation of diverse cellular processes, including control of asexual and sexual

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development, growth morphology, secondary metabolism and virulence (Bayram et al., 2008a).

Although there are just few studies regarding the function of VelB (Bayram et al., 2008a; Chang et al., 2013; Lan et al., 2014; López-Berges et al., 2013; Yang et al., 2013), several researchers have described the function of LaeA and VeA in different fungal species. The nuclear localized LaeA histone methyltransferase was first described in *Aspergillus* spp. as a global regulator of secondary metabolism (Bok and Keller, 2004). VeA was originally discovered in *A. nidulans* as an inhibitor of light-dependent conidiation (Käfer, 1965). It was later reported that the velvet complex and aspects of sexual and asexual development are strongly linked with secondary metabolite biosynthesis (Calvo et al., 2002; Chang et al., 2001; Kato et al., 2003). In an earlier study, Crespo-Sempere et al. (2013b) reported that darkness stimulated *A. carbonarius* secondary metabolite production, whereas light propitiated sexual or asexual development. This effect seemed to be regulated, in part, by the velvet complex and the environmental conditions triggered by light and darkness (Bayram et al., 2008a). The molecular mechanism proposed by Bayram et al. (2008a) elucidated that, in *A. nidulans*, VeA transport to the nucleus is inhibited by light while, in the dark, most VeA protein is found in the nuclei. During darkness, VeA migrates to the nucleus through interactions with other elements that involve an importin α, KapA, and the velvet-like protein B (VelB). In the nucleus, VeA interacts with LaeA and their union triggers secondary metabolism pathways in this species.

Although there is some information about the role of LaeA and VeA in different fungi, nothing has been reported about their function(s) in any *Alternaria* spp. Hence, this is the first work highlighting some processes involving LaeA and VeA in *Alternaria*. For this purpose we have deleted *laeA* and *veA* genes in *A. alternata* and analyzed how these deletions affect growth morphology, asexual development (sporulation and germination), mycotoxin production, virulence when infecting tomato fruit, and expression of genes related to the velvet complex in *A. alternata* (the melanin and the mycotoxin biosynthesis pathways).

2. Material and methods

2.1. Fungal strains, fruit material and growth conditions

The *A. alternata* strains used in this study were CBS 116.329 (isolated from apple), provided by Centraalbureau voor Schimmelcultures (CBS, Utrecht, The Netherlands), and ATCC 66981 (isolated from peanut), kindly provided by C. Lawrence (Virginia Bioinformatics Institute, Virginia, USA). *Agrobacterium tumefaciens* AGL-1 strain was kindly provided by L. Peña (Instituto Valenciano de Investigaciones Agrarias, Valencia, Spain). Both wild-type strains of *A. alternata* were used as controls. For each wild-type strain one knockout of LaeA and one knockout of VeA were used to carry out the experimental assays.

To prepare conidial suspensions, strains were grown on Potato Dextrose Agar plates (PDA; Biokar Diagnostics, France) in the dark at 26 °C for 14 days. Conidia were collected with a scalpel into a sterile solution of 0.005% (v/v) Tween 80 (J.T. Baker, Deventer, The Netherlands) and filtered through Miracloth (Calbiochem, USA). Conidial concentration was determined using a Thoma chamber. Fungal strains were stored as conidial suspensions at −20 °C with 40% glycerol.

For the study of growth infection, wild-types, Δ*veA*, and Δ*laeA* mutants were inoculated in tomato fruit (*Solanum lycopersicum* var. *palladium*). The tomato variety was selected taking into account its susceptibility to *Alternaria* spp. infection.

2.2. Construction of veA and laeA deletion strains

A Blastx algorithm with LaeA nucleotide sequence from *A. flavus*, *A. nidulans* and *Cochliobolus heterostrophus* (Accession numbers AY883016, AY394722 and JF826792) and VeA from *A. flavus*, *A. nidulans* and *C. heterostrophus* (Accession numbers DQ296645, AF109316 and JF826791) as queries was performed in the *A. alternata* genome, recently sequenced by Dang et al. (2015) using a 454 Titanium deep sequencing technology (Roche, Indianapolis, USA). To construct the *laeA* and *veA* gene replacement plasmids (Fig. 1A), 1.7 kb upstream and downstream fragments from the promoter and terminator regions of *laeA* and *veA* genes were cloned into the plasmid vector pRF-HU2 (Frandsen et al., 2008), a binary vector designed to be used with the USER friendly cloning technique (New England Biolabs, USA), as described previously by Crespo-Sempere et al. (2011). The specific primers used for amplifying the promoter and terminator regions were A-VA, A-VB, A-VE and A-VF for *veA* and A-LA, A-LB, A-LE and A-LF for *laeA* (Table 1, Fig. 1B) including vector-specific 9 bp long overhangs containing a single 2-deoxyuridine nucleoside in the 5′ end, which ensured directionality in the cloning reaction. Upstream and downstream fragments were amplified by PCR from genomic DNA of *A. alternata* (CBS 116.329 and ATCC 66981) with DFS-Taq DNA Polymerase (Bioron, Germany). Genomic DNA extraction from mycelium was developed as described in (Crespo-Sempere et al., 2013a). Cycling conditions consisted of an initial denaturation step at 94 °C for 5 min, 35 cycles of 94 °C for 1 min, 58 °C for 1.5 min and 72 °C for 3 min, and a final elongation step at 72 °C for 10 min. Both DNA inserts and the digested vector were mixed together and treated with the USER (uracil-specific excision reagent) enzyme (New England Biolabs, USA) to obtain plasmids pRFHU2-VEA and pRFHU2-LAEA (Fig. 1A). An aliquot of the mixture was used directly in chemical transformation of *E. coli* DH5α cells without prior ligation. Kanamycin resistant transformants were screened by PCR. Proper fusion was confirmed by DNA sequencing using primers A-VD and A-VG for *veA*, A-LD and A-LG for *laeA*, and RF1 and RF2 for both (Table 1). Then, plasmids pRFHU2-VEA and pRFHU2-LAEA were introduced into chemically competent *A. tumefaciens* AGL-1 cells.

Transformation of *A. alternata* was done as described previously by Crespo-Sempere et al. (2011) using *A. tumefaciens* AGL-1 cells carrying the plasmids pRFHU2-VEA and pRFHU2-LAEA. Equal volumes of IMAS-induced bacterial culture (De Groot et al., 1998) and conidial suspension of *A. alternata* (10^6 conidia/mL) were mixed and spread onto nitrocellulose membrane filters (Sartorius Stedim Biotech, Germany), which were placed on agar plates containing the co-cultivation medium (same as IMAS, but containing 5 mM instead of 10 mM of glucose). After co-cultivation at 26 °C for 40 h, the membranes were transferred to PDA plates containing 100 μg/mL of hygromycin B (Calbiochem, USA), as the selection agent for fungal transformants, and 200 μg/mL of cefotaxime (Calbiochem, USA) to inhibit growth of *A. tumefaciens* cells. Hygromycin resistant colonies appeared after 6 to 7 days of incubation at 26 °C. Fig. 1B shows in more detail the gene replacement strategy followed in order to disrupt *veA* and *laeA*.

To ensure a correct deletion of *veA* and *laeA* and the absence of ectopic insertions the conventional PCR and the quantitative PCR (qPCR) were used to determine gene copy number (GC) of the T-DNA inserted in *A. alternata*. Firstly, disruption of *veA* and *laeA* was confirmed by PCR analyses of the transformants (Fig. 1C). The insertion of the selection marker at the correct homologous site was checked with the primer pair A-VI and A-VJ for *veA* and A-LI and A-LI for *laeA* (Table 1). The predicted product in wild-type strains for *laeA* and *veA* was 1892 and 3084 bp, respectively, while for Δ*laeA* strain the DNA band was predicted to be 2658 bp and for Δ*veA* about 2635 bp. To exclude the possibility that the T-DNA was integrated elsewhere in the genome, the qPCR was used instead of a Southern blot analysis. Hence, to determine the number of T-DNA molecules that had been integrated in the genome of each selected transformant, a qPCR analysis was carried out following an already demonstrated methodology described by several authors (Crespo-Sempere et al., 2013c; López-Pérez et al., 2015; Solomon et al., 2008) and firstly described in a filamentous fungus by De Preter et al. (2002). Two primer pairs, (Fig. 1B, Table 1), were designed within the T-DNA in the promoter region of the target genes, close to the selection marker, A-VK and A-VM for *veA* and A-LK and A-LM for *laeA*. qPCR reactions were performed in a final volume of 10 μL, containing 1X of