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Cochliobolus heterostrophus Llm1 – A Lae1-like methyltransferase regulates T-toxin production, virulence, and development

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ABSTRACT

A Lae1-like methyltransferase, Llm1, was identified in maize pathogen *Cochliobolus heterostrophus* which is renowned for production of the secondary metabolite host-selective toxin, T-toxin, and is a model for mechanisms of reproduction of heterothallic Dothideomycetes. Previously, we determined that *C. heterostrophus* mutants lacking Lae1 and Vel1 proteins were decreased in ability to produce T-toxin when the fungus was grown in the dark, demonstrating that these proteins are positive regulators of toxin production. We showed also that Lae1 and Vel1 regulate resistance to oxidative stress and both sexual and asexual reproduction. Here, it is demonstrated that Llm1, one of nine Lae1-like methyltransferases in the *C. heterostrophus* genome, acts as a negative regulator of T-toxin production and thus impacts virulence to the host. *In vitro*, in the dark, and *in planta*, *llm1* mutants make more T-toxin than do wild-type (WT) strains, while overexpressing strains make less than WT. Virulence (amount of chlorosis) to maize, due to T-toxin, follows accordingly. Expression of nine genes involved in T-toxin production is elevated in *llm1* mutants and reduced in overexpressing strains. *llm1* mutations cannot rescue deficiencies in T-toxin production of *lae1* or *vel1* mutants indicating that Llm1 represses T-toxin biosynthesis, and that *vel1* and *lae1* mutations are epistatic to *llm1* mutations. Thus, increased T-toxin production, and presumably gene expression, in the *llm1* mutant is dependent on the presence of Vel1 and Lae1 proteins. There is no evidence that Llm1 has an effect on oxidative stress tolerance. *llm1* mutants are fully fertile in crosses to WT mating testers, while *LLM1* overexpressing strains and *llm1lae1* and *llm1vel1* double mutants are unable to act as females. Overexpression of *LLM1* leads to de-repression of asexual sporulation during sexual development, and of asexual sporulation in the light and the dark during vegetative growth, as is the case for *vel1*, *llm1vel1*, and *llm1lae1*-deletion strains. *llm1vel1* and *llm1lae1* double mutants are similar to *lae1* single mutants and accumulate more hyphal melanin in liquid medium than do *llm1* or *vel1* single mutants, implying Llm1 plays a redundant role in regulating pigmentation with Vel1, while Lae1 plays a major role.

1. Introduction

Cochliobolus heterostrophus, a necrotrophic maize pathogen, and causal agent of Southern Corn Leaf Blight, is found in nature as one of two races. Race T (Smith et al., 1970; Ullstrup, 1972) is highly virulent on Texas male sterile cytoplasm (T-cms) corn due to production by the fungus of the secondary metabolite, host selective toxin, T-toxin, and the presence of its target, the inner mitochondrial membrane protein, URF13, in the T-cms host (Braun et al., 1990; Levings et al., 1995). Race O, which lacks ability to biosynthesize T-toxin, is only mildly virulent to T-cms corn. To date, nine genes associated with T-toxin production, unique to race T have been characterized (Baker et al., 2006; Inderbitzin et al., 2010; Rose et al., 2002). These genes have complex genome architecture and phylogenetic relationships; they are not clustered like many secondary metabolite genes but distributed in disconnected fragments on two different chromosomes (Kodama et al., 1999); only two share a common evolutionary history (Inderbitzin et al., 2010).

Two *C. heterostrophus* proteins, Lae1 (a methyltransferase) and Vel1 (a velvet family protein), homologs of *Aspergillus nidulans* LaeA and VeA, the first such proteins to be described, have been demonstrated to positively regulate expression of the genes (*Tox1*) associated with production of T-toxin, in response to light (Wu et al., 2012). To date these are the only proteins known to regulate *Tox1*. Expression of all nine known *Tox1* genes is induced in the dark and repressed in the light resulting in increased production of T-toxin in the former and minimal production in the latter (Wu et al., 2012). Overexpression (OE) of *LAE1* in light induces *Tox1* expression, compared to that in dark, indicating that control of Lae1 levels is crucial for light-responsive stimulation of T-toxin production (Wu et al., 2012). Lae1 and Vel1 proteins affect both T-toxin mediated high virulence and general pathogenicity to maize. The effect of these proteins on pathogenicity was demonstrated recently for *Botrytis cinerea* (Schumacher et al., 2012).

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C. heterostrophus Lae1 and Vel1 also play positive roles in regulation of oxidative stress and sexual differentiation in the dark; deletion of the corresponding genes leads to hypersensitivity to oxidative stress and inability to act as females in crosses (Wu et al., 2012). Lae1 and Vel1 negatively regulate asexual sporulation and melanin production (Wu et al., 2012).

Cumulative research on velvet family proteins such as VeA/Vel1 has established that they collaborate with the Lae1/LaeA protein to regulate secondary metabolism and morphological development, in response to light, in numerous filamentous fungi (Amaike and Keller, 2009; Bayram et al., 2008a; Calvo et al., 2002, 2004; Bayram and Braus, 2012; Wiemann et al., 2010; Hoff et al., 2010; Wu et al., 2012; Yang et al., in press; Schumacher et al., 2012). All members of the velvet family possess a conserved putative protein–protein interaction domain (Sarikaya Bayram et al., 2010). In the velvet protein interaction network of *A. nidulans*, VeA links another velvet protein, VelB, to LaeA in the dark to regulate secondary metabolism. VelB interacts also with an additional velvet member, VosA, to repress asexual development in the dark (Sarikaya Bayram et al., 2010).

LaeA, a methyltransferase with a nuclear localization signal at the N-terminus was initially recognized as a secondary metabolite gene transcriptional regulator (Bok and Keller, 2004; Hoff et al., 2010; Wiemann et al., 2010), then shown to be involved in development and control of VeA modification and protein levels (Bayram et al., 2008a; Sarikaya Bayram et al., 2010). LaeA is pivotal in formation of VelB–VeA–LaeA (Bayram et al., 2008a) and VelB–VosA complexes (Bayram et al., 2010). It has been speculated (Sarikaya Bayram et al., 2010) that there is competition for VelB between the VelB–VosA and VelB–VeA–LaeA complexes. In the absence of LaeA, the amount of VelB–VosA complex increases resulting in elevated repression of asexual development in dark (Sarikaya Bayram et al., 2010).

Additional methyltransferases, termed LaeA-like methyltransferases, are present in fungal genomes (Palmer et al., 2011, in press). One of these, *A. nidulans* LlmF, has been reported to negatively regulate secondary metabolism and sexual development, associating LlmF with the opposite regulatory role from that of LaeA (Palmer et al., 2011, in press). LlmF is essential to subcellular localization of VeA.

Here, we mined the *C. heterostrophus* genome to identify the entire suite of LaeA-like methyltransferases (Llm) then explored function of one of these, Llm1, an ortholog of *A. nidulans* LlmF. We found Llm1 negatively regulates T-toxin production and concomitant chlorosis on host plants, and thus has the opposite role of Lae1 in this regard. Loss of Llm1 has no effect on sexual or asexual reproduction. During vegetative growth overexpression of *LLM1* increased asexual sporulation and strains had fewer aerial hyphae regardless of light conditions. During sexual reproduction overexpression strains were incompetent as females and asexual sporulation was de-repressed. Lack of, or overexpression of, *LLM1* has no effect on management of oxidative stress or control of melanization, in contrast to loss of *LAE1*. In addition, we constructed *llm1lae1* and *llm1vel1* double mutants to determine epistatic relationships. Vel1 and Lae1 are epistatic to Llm1 with regard to regulation of T-toxin production and asexual development and sporulation during vegetative reproduction.

2. Materials and methods

2.1. Fungal strains, plant materials, and general growth conditions

C. heterostrophus strains C4 (*Tox1*$^+$; *MAT1-2*, ATCC 48331), C5 (*Tox1*$^-$; *MAT1-1*, ATCC 48332), CB7 (*Tox1*$^+$; *MAT1-1*; *alb1*), CB11 (*Tox1*$^+$; *MAT1-2*; *alb1*), 1733-R-3 (*MAT1-1*; *alb1*; *lae1*) and 1732-T1-1 (*MAT1-1*; *alb1*; *vel1*) were used (Leach et al., 1982; Oide et al., 2010; Wu et al., 2012). *LLM1* was deleted in strain C4. *LLM1* complemented strains were obtained by introducing a PCR generated fragment of the WT *LLM1* coding, upstream, and downstream flanking sequences to *llm1*-deletion strain *llm1*-B1 by co-transformation with plasmid pNG carrying the Neomycin Phosphotransferase II (*NPTII*) gene which confers resistance to G418 (Oide et al., 2007; Wu et al., 2012). Double mutant strain 1758-R-26 (*MAT1-1*; *llm1*; *lae1*) is a random (R) progeny isolated from a cross between *llm1* mutant strain, *llm1*-B1 and *lae1* mutant strain 1733-R-3. Double mutant strain 1759-R-12 (*MAT1-1*; *llm1*; *vel1*) is a progeny isolated from a cross between *llm1* mutant strain *llm1*-B1 and *vel1* mutant strain 1732-T1-1. Unless mentioned otherwise, all strains were grown on complete medium with xylose (CMX) (Inderbitzin et al., 2010) at 23 °C under constant light except for experiments involving OE strains which were grown on CMX supplemented with polygalacturonic acid (PGA) (Wu et al., 2012).

Zea mays cultivars W64A-N and W64A-T, used for all virulence tests, were propagated in a growth chamber with a light regimen of 16 h of light/8 h of dark at 24 °C.

2.2. Identification of the LLMF ortholog in C. heterostrophus

A. nidulans LlmF (ANID_06749.1) amino sequence was used to query the *C. heterostrophus* race T strain C4 sequence database (http://genome.jgi-psf.org/CocheC4_1/CocheC4_1.home.html); all hits with an *E* value $<1.0 \times e^{-5}$ were extracted. The *A. nidulans* LlmF sequence was also used to query the *A. nidulans* database (http://genome.jgi-psf.org/Aspnid1/Aspnid1.home.html) and all hits with an *E* value $<1.0 \times e^{-5}$ were extracted. In addition, all *Cochliobolus* spp. genome sequences and the *Setosphaeria turcica* genome sequence on the JGI Mycocosm website (http://genome.jgi-psf.org/programs/fungi/index.jsf), were queried with the top *C. heterostrophus* hit (ID # 45679) and the *A. nidulans* LlmF (ANID_06749.1) protein and the best match extracted. Both query proteins yielded the same top hits in the additional genomes. An amino acid alignment of these sequences was generated using ClustalW and phylogenetic trees were built based on the maximum likelihood principle using web-server RAxML BlackBox (http://phylobench.vital-it.ch/raxml-bb/) (Stamatakis et al., 2008). Bootstrap values were calculated on 1000 replicates. The ProtTest server was used to decide the best-fit protein evolutionary model (Abascal et al., 2005). Clades supported with 100% bootstrap values and containing a unique protein from each species were collapsed using the FigTree v1.3.1 program (http://tree.bio.ed.ac.uk/software/figtree).

2.3. Construction and PCR verification of gene deletion, complementation, and overexpression strains

The split-marker deletion method (Catlett et al., 2003) was improved to construct *LLM1* deletions. In the modified approach, the entire selection marker fragment "*hygB*", including the promoter, was amplified from plasmid pUCATPH using M13R and M13F primers, then fused to the 5' and 3' flanking fragments of the target *LLM1* gene. Transformation protocols are described in Turgeon et al. (2010). Primer sequences used for gene deletion, complementation, and overexpression are listed in Table S1 and corresponding agarose gel images verifying these manipulations are shown in Fig. S1.

Co-transformation, described previously (Wu et al., 2012), was used to construct *llm1*[*LLM1*] strains complemented at the native *Llm1* locus. A *LLM1* OE vector, pHPLLM1, carrying the *LLM1* coding region driven by the polygalacturonic acid (PGA)-inducible PelA promoter from *A. nidulans* (Yang et al., 1994) was constructed and transformed into WT strain C4 as described previously (Turgeon et al., 2010; Wu et al., 2012). Integrations of all constructs