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Role of the *Alternaria alternata* Blue-Light Receptor LreA (White-Collar 1) in Spore Formation and Secondary Metabolism

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***Alternaria alternata* is a filamentous fungus that causes considerable loss of crops of economically important feed and food worldwide. It produces more than 60 different secondary metabolites, among which alternariol (AOH) and altertoxin (ATX) are the most important mycotoxins. We found that mycotoxin production and spore formation are regulated by light in opposite ways. Whereas spore formation was largely decreased under light conditions, the production of AOH was stimulated 2- to 3-fold. ATX production was even strictly dependent on light. All light effects observed could be triggered by blue light, whereas red light had only a minor effect. Inhibition of spore formation by light was reversible after 1 day of incubation in the dark. We identified orthologues of genes encoding the *Neurospora crassa* blue-light-perceiving white-collar proteins, a cryptochrome, a phytochrome, and an opsin-related protein in the genome of *A. alternata*. Deletion of the white-collar 1 (WC-1) gene (*lreA*) resulted in derepression of spore formation in dark and in light. ATX formation was strongly induced in the dark in the *lreA* mutant, suggesting a repressing function of LreA, which appears to be released in the wild type after blue-light exposure. In addition, light induction of AOH formation was partially dependent on LreA, suggesting also an activating function. *A. alternata* Δ*lreA* was still able to partially respond to blue light, indicating the action of another blue-light receptor system.**

Alternaria is a genus of filamentous ascomycetes containing many species of economic importance, including saprophytes, phytopathogens, and zoopathogens. Many *Alternaria* species are potent producers of mycotoxins and allergens, properties which may directly affect human life. Every year, large amounts of food and feed are contaminated and destroyed because of *Alternaria* growth and toxin production. Worldwide epidemiological studies indicate that *Alternaria* sensitivity is closely linked with the development of asthma (1). Despite the great importance of *Alternaria* species, little is known about the molecular biology underlying *Alternaria* biology.

A. alternata is a widespread saprophytic species which produces a wide variety of different secondary metabolites, among which are the mutagenic mycotoxins alternariol (AOH) and altertoxin (ATX) (2–4). For AOH and its monomethylether (AME), it has been shown recently that they have potential to cause DNA double-strand breaks in mammalian cells *in vitro* (5). The altertoxins ATX-I, -II, and -III are mutagenic in the Ames test and are more potent and acutely toxic to mice than AOH and AME (6). Surprisingly, it is not clear yet how AOH or ATX is produced or how their production is regulated. Alternariol biosynthesis requires the activity of a polyketide synthase, 10 of which were identified recently in the genome. One of them, PksJ, was identified to catalyze the initial steps of alternariol biosynthesis (7). It was already reported 30 years ago that blue light reduced alternariol production in *A. alternata* (8). Blue light has also been reported to inhibit sporulation in *A. tomato* and *A. cichorii*. The effect was, in addition, temperature dependent and occurred only in mature conidiophores (9). These observations suggest that *Alternaria* species are able to sense and respond to light. It has been well established, meanwhile, that many fungi are able to respond to light and employ one or several photoreceptors (10–12). The existence of light receptors in filamentous fungi has been shown for *Neurospora crassa*, *Aspergillus nidulans*, *Coprinus cinereus*, *Cryptococcus neoformans*, *Phycomyces blakesleeanus*, and *Physarum polycephalum* and for many others. Some of them possess receptors for blue, red, and green light and are therefore able to sense light over a broad spectral range. The perception of light in these fungi has been shown to influence asexual conidiation, sexual development, pigmentation, the circadian clock, and secondary metabolism (10, 12). The blue-light response often seems to be the most important one; e.g., all light-dependent processes in *N. crassa* are regulated by UV or blue light (13, 14). In contrast, *A. nidulans* responds well to red light in addition to blue light (15, 16). It was discovered recently that the blue- and red-light-sensing chromoproteins along with some additional proteins form a light-regulator complex in this fungus (17).

Here we studied the effect of light on the regulation of spore formation and mycotoxin production in *A. alternata* and assigned a function to LreA, the white-collar 1 (WC-1) orthologue, as the main photoreceptor in this fungus.

MATERIALS AND METHODS

Culture conditions and harvesting of spores. *Alternaria alternata* DSM 12633 cultures were grown on modified Czapek Dox broth (mCDB) agar if not stated differently and incubated 1 to 7 days at 28°C. For white-light experiments, a 10-W energy-saving lamp (Flair energy) was used; for red-

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and blue-light conditions, light-proof, ventilated boxes with wavelength (450- and 680-nm)-specific LEDs were used. The intensities of the blue- and red-light-emitting diodes (LEDs) were measured at 0.72 W/m^2 and 1.45 W/m^2, respectively.

All plates were inoculated with 5×10^4 spores. For quantification, the spores were harvested in sterile H_2O, filtered for separation from the mycelium, and centrifuged. The pellet was resuspended in 5 ml H_2O, and the optical density at 600 nm (OD_{600}) was measured with a spectrophotometer (Pharmacia Ultraspec III). The relation of the OD_{600} to the amount of spores was calculated before via counting spores in a Helber chamber. All experiments were done with three technical replicates.

Analysis of mycotoxins using TLC and LC/MS. For the extraction of mycotoxins, three disks from each plate were excised with the back of a blue pipette tip and extracted by shaking with 1 ml ethyl acetate for 1 h. The solvent was vaporized in a SpeedVac and the pellet resolved in 60 μl ethyl acetate. A 20-μl volume was used for thin-layer chromatography (TLC) with a mobile phase composed of toluole, ethyl acetate, and formic acid (5:4:1) on silica plates (Merck TLC silica gel 60) and visualized under UV light at 365 nm. As a standard, TLC-prepared AOH was used. For liquid chromatography-mass spectrometry (LC/MS) analysis, the same extracts were used but the ethyl acetate was evaporated and the pellet resolved in 100 μl methanol.

LC-DAD-MS analysis. A LXQ Linear Ion Trap multiple-stage MS (MSn) system (Thermo Fisher Scientific, Waltham, MA) together with a Finnigan Surveyor high-performance LC (HPLC) system equipped with a binary pump, autosampler, diode array detector (DAD), and Xcalibur 2.0.7 software for data collection and analysis was used. This allowed on-line analysis of UV absorption and MS. Extracts (10 μl) were injected. Separation was carried out on a 5-μm-pore-size, reversed-phase Luna C_8 column (Phenomenex, Torrance, CA) (250 mm by 4.6 mm inside diameter [id]). Solvent A was deionized water, and solvent B was acetonitrile (CAN). A gradient was started at 30% B and was changed from 30% B to 100% in 20 min. After the column was eluted with 100% B for 10 min, the initial 30% B was reached in 3 min. The flow rate was 0.5 ml/min. The mass spectrometer was operated in the negative electrospray ionization (ESI) mode. Nitrogen was used as the sheath gas, auxiliary gas, and sweep gas, with flow rates of 40.0, 10.0, and 0.02 liters/min, respectively. Spray voltage was 5.5 kV, spray current 0.05 μA, capillary voltage −1.0 V, capillary temperature 300°C, and tube lens voltage −69.89 V. For MSn analysis, the collision-induced dissociation (CID) voltage was set to 1.75 V.

Generation of an *lreA* deletion strain by fusion PCR. For the fusion of *lreA* upstream and downstream regions to a selection marker cassette, the protocol of Szewczyk and coworkers was essentially followed (18). One-kilobase upstream and downstream regions of the *lreA* open reading frame (ORF) were amplified with primers wc1_LB_fw_P1, wc1_LB_rv_P3, wc1_RB_fw_P4. and wc1_RB_rv_P6. P3 and P4 contain overhangs complementary to the ends of the hygromycin B cassette used as the selection marker and amplified with primers hph_sfia_fw_neu2 and hph_sfib_rv_neu2 from the pPK2 vector (kindly provided by N. Requena, Karlsruhe, Germany). The sequences of all primes are listed in Table 1. The cassette consists of the *gpdA* promoter from *A. nidulans*, the hygromycin phosphotransferase gene from *Escherichia coli*, and the *trpC* terminator from *A. nidulans*. The borders and cassette were amplified via fusion PCR in one step with nested primers wc1_P2 and wc1_P5. As the polymerase, PrimeStar polymerase (TaKaRa Bio Inc., Japan) was used. PCR conditions were as follows: an initial denaturing step at 98°C for 2 min, followed by 25 cycles of 10 s of denaturation at 98°C, 7 s of annealing at 60°C, 55 s of elongation at 72°C with an increase of 10 s per cycle, and a final elongation at 72°C for 7 min. The purified product was directly used for transformation.

For control PCRs, *Taq* polymerase was used with primers wc1_up_LB_fw2 and Hyg_gpd_rv for the left border and Hyg_end_fw and wc1_p6 for the right border. Southern blot analyses were performed following standard procedures (19).

TABLE 1 Primers used in this study[a]

Primer	Sequence[a]
wc1_LB_fw_P1	TCCAGAGGGAAGTGACATGG
wc1_P2	GAGCAACTCCAAGGTAACGTC
wc1_LB_rv_P3	*CGTATTTCAGTGTCGAAAGATC*TGTAAGGTTGGGATGCGAAG
wc1_RB_fw_P4	*CCCACTCCACATCTCCACTCGA*TTTTCCTCTTCTGTTTCGTGTC
wc1_P5	ACGTTCCTCATTCTGCTTCC
wc1_RB_rv_P6	GCCCCCCAATCTAGTTGTAAT
hph_sfia_fw_neu2	GGCCATCTAGGCCCCGGGGATCTTTCGACACTGAAATACG
hph_sfib_rv_neu2	GGCCTGAGTGGCCGGTCGAGTGGAGATGTGGAGTGGG
wc1_up_LB_fw2	CTGTCTTGTCTCCGTCTTTG
hyg_gpd_rv	CTCGACGTATTTCAGTGTCG
hyg_end_fw	GTCCGAGGGCAAAGGAATAG
pksC_RT_fwd	GCCAATGAAGACGGCCAC
pksC_RT_rev	CCGATCCAACTGTGTTCAG
pksD_RT_fwd	CGTGTTTGTTGGCACGATG
pksD_RT_rev	GAGATCTACTGCGATCATGC
pksA_RT_fw	AGTTCCGCCCGCTATCGCTCGTC
pksA_RT_rv	GTAACGCCAATCGCGGATACCGAGCAAT
J_RT_fwd_N	GTCCCAAATTCCTACCCTCAC
J_RT_Rv_N	GATAGCCATCGAAAGCATTCCC
lreA_P1	GTGACTTCAAGAGAGATGATG
lreA_P2	TCCAGAGGGAAGTGACATGG
lreA_P3	*GTAGTTGGCATTGGCTTTCCCTAGGCCC*CCCAATCTAGTTGTAATC
nptII_P4	*CCTAGGGAAAGCCAATGCCAACTAC*GACGTTAACTGATATTGAAGGAG
nptII_P5	AACCCAGGGGCTGGTGA
nptII_P6	GCTGAGGAACTTGCAAAGCA

[a] Orientation, 5′→3′. Overhangs and restriction sites are indicated in italics.

Protoplast transformation of *A. alternata*. The transformation procedures based on the protocol of *A. brassicicola* (20) were used, with modifications. Fungal spores were harvested from a mCDB culture plate, filtered, and inoculated into 100 ml Richard's liquid medium (sucrose at 20 g/liter, KNO_3 at 10 g/liter, KH_2PO_4 at 5 g/liter, $MgSO_4 \cdot 6H_2O$ at 2.5 g/liter, yeast extract at 1 g/liter) for 19 to 24 h at 30°C and 150 rpm. The mycelium was harvested by filtering, washed with 0.7 M NaCl, and digested in a Kitalase (Wako Chemicals) suspension (60 mg in 6 ml 0.7 M NaCl) for 1 h with soft shaking at 80 rpm and 30°C. Protoplast quality and quantity were checked via microscopy. Protoplasts were separated by filtering through miracloth and glass wool and washed with 0.7 M NaCl by centrifugation at 7,000 rpm and 4°C for 10 min, followed by a second washing step with STC (1 M sorbitol, 50 mM $CaCl_2$, 50 mM Tris-Cl, pH 8.0). The pellet was resuspended in 200 to 500 μl STC, and protoplasts were counted in a Helber chamber. Protoplasts (4×10^6) were prepared for DNA absorption by 1 min of incubation at 37°C. A 4-μg volume of DNA was added, and the mixture was incubated on ice for 30 min. Cells were subjected to heat shock for 2 min at 42°C and, after addition of 2 ml PEG (40% polyethylene glycol [PEG] 4000, 50 mM Tris-Cl [pH 8.0], 50 mM $CaCl_2$), incubated for 20 min at room temperature. The suspension was spread on regeneration medium containing hygromycin B (HygB) (1 M sucrose, 0.5% caseic acids, 0.5% yeast extract, 80 μg/ml HygB) and incubated for 3 days at 28°C.

Purification of transformants. Transformation results were confirmed via PCR and Southern blot analysis with probes against hygromycin phosphotransferase and *lreA*. Because only heterokaryotic strains were obtained, strains were purified via 3 rounds of single-spore isolation and 1 round of protoplast isolation.

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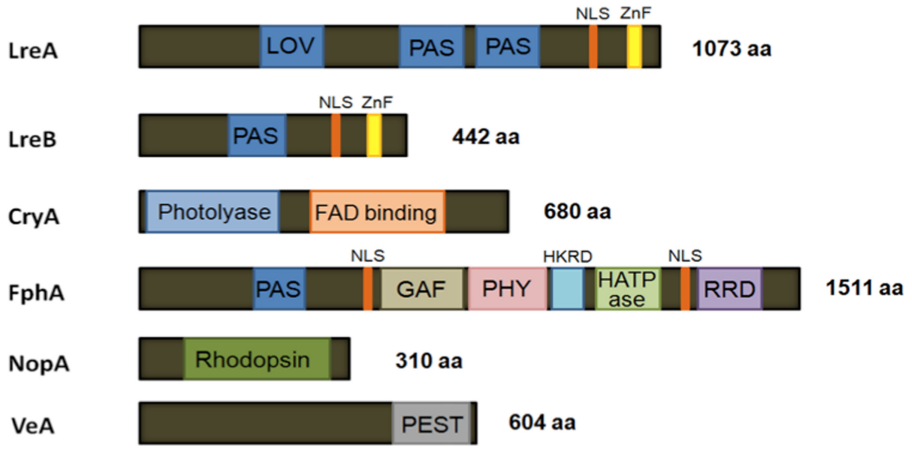

FIG 1 Scheme of *A. alternata* proteins involved in light regulation in other fungi. Predicted intron borders were not confirmed experimentally. The number of amino acids is given to the right of each protein structure. The protein sequences were derived from the genomic sequence.

Expression analysis. Cultures were grown for 2 days in the dark in static liquid cultures at 28°C and subsequently for 1 h in blue light (450-nm wavelength). The mycelium was dried, frozen in liquid nitrogen, and crushed with glass beads in buffer RLT (Qiagen, Hilden, Germany) (without mercaptoethanol) using a Retsch MM 200 mixer mill. RNA was extracted with a Qiagen RNeasy Plant minikit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Gene expression in the wild-type strain and in the transformants was analyzed by quantitative real-time PCR in an iCycler iQ detection system from Bio-Rad. For expression analysis of the polyketide synthases, cultures of the wild-type and transformant strains were incubated for 7 days in the dark or in white light in static liquid cultures at 28°C. The mycelium was dried, frozen in liquid nitrogen, and crushed. RNA was then extracted with a Qiagen RNeasy Plant minikit (Qiagen, Hilden, Germany). Primers are listed in Table 1.

Recomplementation of the *lreA* deletion strain. For the recomplementation of the Δ*lreA* strain, a fusion construct was used. First, *lreA*, including the putative promoter and terminator regions and a linker, was amplified with primers lreA_P1 and lreA_P3. The Geneticin resistance gene was amplified with primers nptII_P4, which also contains the linker, and nptII_P6. With primers lreA_P2 and nptII_P5, a fusion product of *lreA* and the Geneticin resistance gene was amplified. This PCR product was directly used after a purification step for transformation. A total of 12 transformants growing in the presence of the aminoglycoside antibiotic Geneticin were tested by PCR, and 1 was shown to be positive for *lreA*. Whether the strain was a heterokaryon or whether the original knockout cassette was replaced was not tested.

RESULTS

The *A. alternata* genome harbors orthologues of all known fungal photoreceptor proteins. The *A. alternata* genome has recently been sequenced using 454 deep sequencing technology, and a first draft of the genome sequence has been used to search for orthologues of proteins known from other fungi to be involved in light signaling. The complete genome sequence will be published elsewhere. Applying standard BLAST searches, orthologues for all three types of fungal photoreceptors, i.e., for the three light qualities red, blue (and UV), and green, have been identified. In detail, there are orthologues of the white-collar 1 and 2 proteins, one orthologue of a cryptochrome, a phytochrome, and an opsin-related protein. This composition of photoreceptors resembles that in *A. nidulans* (12). In contrast, *N. crassa* harbors two phytochromes and the zygomycetes *Phycomyces blakesleeanus* and *Mucor circinelloides* employ several white-collar orthologues for blue-light perception (10, 21, 22). Therefore, the *A. alternata* proteins are mainly compared here to the ones from *A. nidulans*. The domain structures of the *A. alternata* photoreceptors have been analyzed with SMART, WoLFPSORT, epestfind, ELM, ScanProsite, and InterPro Scan software tools using standard parameters (Fig. 1). The putative *A. alternata* phytochrome is 1,511 amino acids (aa) long and harbors in the N-terminal part a PAS domain, a GAF domain, and a PHY domain and in the C-terminal part a histidine-related kinase domain, a HATPase domain, and a response regulator domain. As in *A. nidulans*, it contains two nuclear localization signals (NLS). The cysteine in the PAS domain of the phytochrome of *A. nidulans* is conserved in *A. alternata*, suggesting functionality of the protein. Following the nomenclature of *A. nidulans*, we named the white-collar proteins light regulator protein A (LreA) and LreB, respectively. LreA comprises 1,073 amino acids and harbors a LOV domain, two PAS domains, a GATA-type zinc finger domain, and a NLS. In *N. crassa* WC-1 and in many orthologues of this protein in other fungi, the chromophore is attached to a conserved cysteine residue in the LOV domain. This residue is also conserved in the *A. alternata* WC-1 homologue (data not shown). LreB is 442 amino acids long and contains a PAS domain and also a GATA-type zinc finger as well as a NLS. As a third blue-light receptor homologue, we found a putative cryptochrome with 680 aa, comprising a photolyase and a FAD binding domain, like the one in *A. nidulans* (23). The possible green-light receptor homologue NopA harbors a rhodopsin domain and is 310 aa long. The function of this protein is not well analyzed in *A. nidulans*, and it is not yet clear whether light perception via this protein is possible at all (our unpublished studies). In addition to the light receptors, we found a 604-aa homologue to the VeA protein which coordinates light signaling with fungal development and secondary metabolism in *A. nidulans* by taking part in a complex with the light receptors FphA, LreA, and LreB as well as in the velvet complex with VeB and LaeA (24–27). It contains a potential PEST domain like that in *A. nidulans* VeA, but a nuclear localization signal (NLS) or nuclear export signal (NES) was not found.

Blue light inhibits spore formation. Previous reports suggested that sporulation in *A. alternata* is light dependent and that the effective quality is blue light (28). In order to confirm this and

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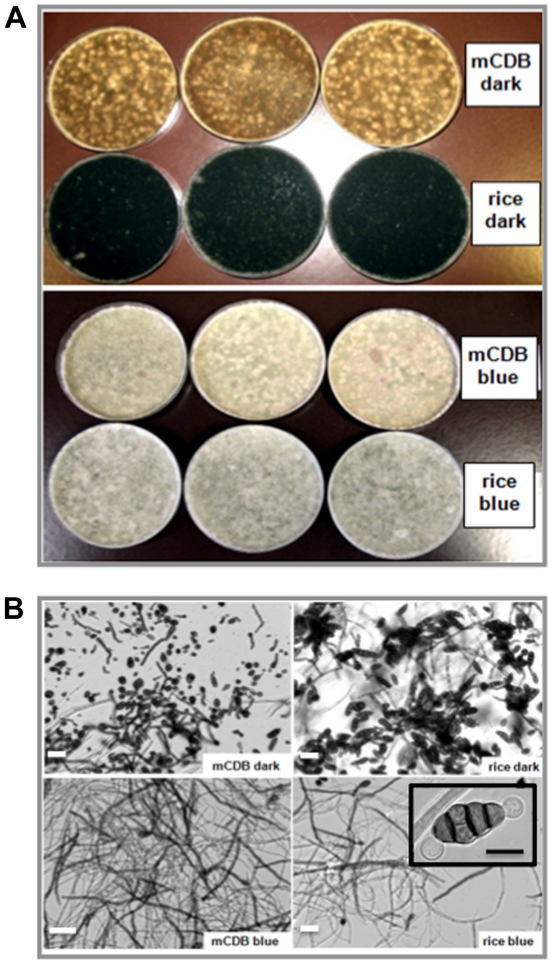

FIG 2 Inhibition of sporulation in blue light on two different media. (A) The upper row shows cultures grown for 6 days in constant darkness and the lower row cultures grown for 6 days in constant blue light. Growth is faster on rice flour agar. In blue light, formation of aerial hyphae is favored and sporulation is strongly inhibited. (B) Microscopic pictures of the colonies on top. The inset shows a multicellular spore in the process of germination. Bar, 20 μm (inset bar, 5 μm).

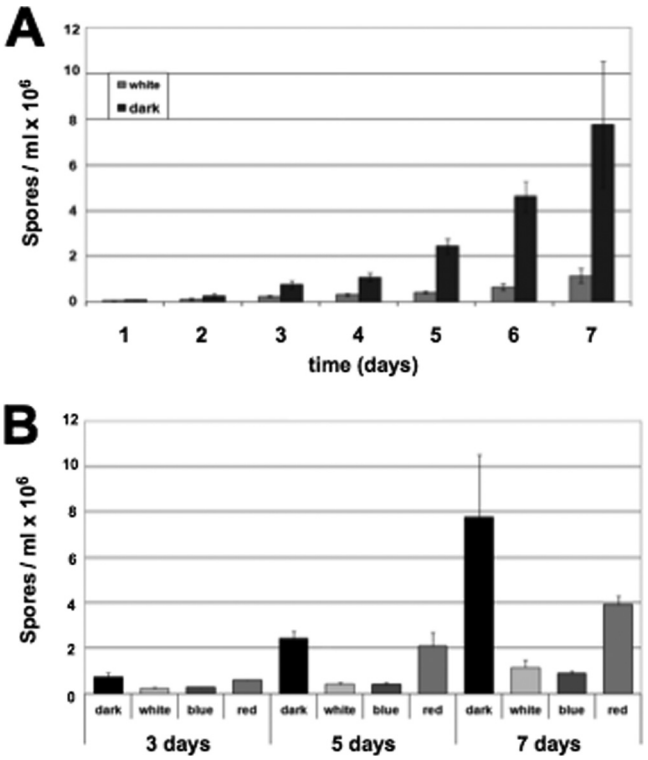

FIG 3 Spore numbers produced in different light qualities. (A) Cultures were incubated on mCDB agar plates for 7 days in constant white light or constant darkness at 28°C. Spores were harvested and counted every 24 h. (B) The same experiment with additional cultures in constant blue and constant red light. Blue- and white-light cultures show similar spore numbers, as do red-light and dark cultures. The difference in the red and dark samples on day 7 may be due to the large standard deviation in the dark samples.

bring it into relation to the putative photoreceptors in *A. alternata*, we studied the effect of light on sporulation. We inoculated agar plates with a defined number of conidiospores and incubated the plates for 1 to 7 days in constant white light as well as constant dark and, later, additionally in blue (450 nm) and red light (680 nm). The cultures showed different colors of the mycelia, and a view under the light microscope revealed a drastic decrease of spore numbers on the plates incubated in constant blue light in relation to dark (Fig. 2). The experiment was done with two different media, mCDB and rice flour medium. The effect was the same on both, but because the fungus grows faster on rice flour medium, it produced more spores in the same time period. The larger number of spores in the dark-grown cultures was already visible by the naked eye, because the spores are black pigmented and thus the agar plates appear dark. To quantify this inhibitory effect, we harvested the spores and determined their numbers via optical density measurements (Fig. 3A). Blue light had nearly the same effect as white light, whereas the effect of red light was similar to that of dark conditions, with the exception of day 7 (Fig. 3B).

Further on, we were interested in the time of blue-light exposure that is necessary for the inhibition of sporulation and tested different exposure times from 1 min to 24 h. Before the cultures were exposed to blue light, they were grown for 2 days in the dark, and after irradiation, the plates were put back in the dark for 18 h. A significant inhibition occurred after 2 or more h of blue-light irradiation (data not shown).

To address the issue of whether the inhibition effect persists or is reversible in the dark, we compared cultures that were incubated for 4 days in constant blue light or for 4 days in constant darkness to cultures that were grown for the first 3 days in blue light and afterward for 1 day in the dark. Whereas the blue-light cultures showed the expected sporulation reduction of about 50%, the sporulation in the dark-after-blue-light culture was nearly fully restored (92%; data not shown). This reversibility is not surprising if it is assumed that the phenotypic effects are caused by a blue-light regulator acting in the way WC-1 does.

Blue light stimulates mycotoxin production. Because *A. alternata* is known as producer of a large spectrum of secondary metabolites, we analyzed the effect of light on the amount of secondary metabolites by thin-layer chromatography (TLC). Häggblom and Unestam described an inhibitory effect of blue light on the production of alternariol (AOH) and alternariol monomethyl ether (AME) in 1979 (8). We inoculated mCDB agar plates in the way described above and incubated them for 7 days. Both mycelium and agar medium were extracted with ethyl acetate and an-

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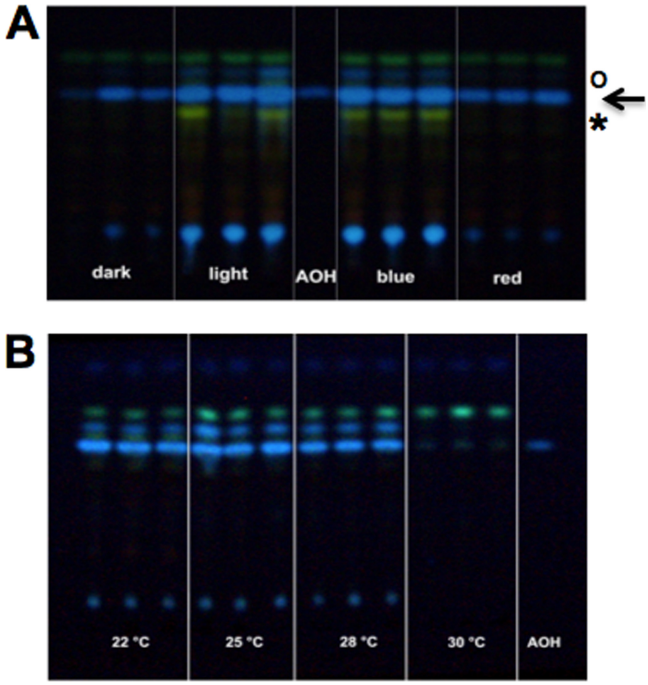

FIG 4 Thin-layer chromatography analysis of secondary metabolites in dependence of light and temperature. (A) Extracts from cultures grown for 7 days on mCDB agar at 28°C under conditions of constant darkness and of white, blue, and red light, respectively. AOH is marked by an arrow, the circle marks AME, and the asterisk marks ATX-I. The latter two are visible only under white- and blue-light conditions. For AOH, the amount is increased under these conditions. (B) Cultures grown 7 days on mCDB agar in darkness at different temperatures. AOH and AME are produced in the same amounts at 22, 25, and 28°C, but the production of both was strongly reduced at 30°C. Growth was not reduced at 30°C.

alyzed by TLC. It was confirmed that the dry weights of dark and blue-light cultures were the same. AOH was identified with a TLC-prepared standard, and the yellow band running below the AOH band could be characterized as altertoxin I (ATX-I) in a LC/MS analysis. In addition, AME and ATX-II were identified, although for ATX-II the proposed structure relies as yet only on the *m/z* values and the UV absorption spectrum. All substances visible on our TLC plates were more abundant in white- and blue-light-grown cultures, although the increases were different for different substances (Fig. 4A). The red-light extracts showed results very similar to those from the dark samples. Whereas AOH was already produced under constant dark conditions, ATX-I production strictly depended on light. The same results were obtained for extracts from cultures grown on rice flour agar (data not shown). The AOH amount on the TLC plates was quantified with InfinityCapt software. Considering the peak volume, AOH showed a 2-fold increase in blue light compared to the dark sample.

AOH and AME production is temperature regulated. Because the parameters for mycotoxin production in fungi may be divers, we checked, among other factors, the influence of the temperature on alternariol production. We tested four incubation temperatures and found no difference in the AOH or AME production when the strain was incubated at 22, 25, or 28°C. At 30°C, AME production was completely and AOH production nearly completely lost (Fig. 4B).

Deletion of the *lreA* white-collar homologue. To start the molecular analysis of light regulation in *A. alternata*, the role of *lreA*, the white-collar 1 orthologue, has been studied by gene deletion. One-kilobase *lreA* upstream (left) and downstream (right border) regions were PCR amplified and fused to the hygromycin B (*hph*) resistance cassette by fusion PCR (18). The 5.9-kb-long PCR product (4 μg) was directly used for protoplast transformation and homologous replacement of the *lreA* open reading frame (Fig. 5A to C). Transformants were analyzed by PCR and Southern blotting. The integration of the construct into the genome was proven by PCR with primers for the 1-kb *hph* gene (data not shown). Homologous integration of the construct was demonstrated by PCR using primers derived from the hygromycin B cassette and primers outside the left or the right border sequences (Fig. 5D). For two of the three transformants, the PCR results showed the expected bands for both sides. Southern blot analysis with a probe for hygromycin B confirmed the results (data not shown). However, a wild-type copy of *lreA* was still detectable by PCR and Southern blotting in all 3 transformants (data not shown). We anticipated that the transformants still harbored non-transformed wild-type nuclei as a heterokaryon. The other possibility of multiple copies in the genome was excluded because there was only one band detected in the Southern blot. To purify the mutants, 3 rounds of single-spore isolation were performed with no effect concerning the existence of the wild-type band. Therefore, protoplasts were generated and regrown to single colonies, 18 of which were further analyzed. In two of them, the wild-type band in the PCR with *lreA* primers was lost and the mycelium of these two strains appeared much darker on agar plates than that of the wild type (Fig. 5B, no. 4 and 5). The other 16 analyzed strains still showed the wild-type signal in the PCR and essentially the same phenotype as the wild type, as shown for no. 2 and 6 in Fig. 5B. For further studies, strain no. 4 was chosen. In Southern blot analyses with the *lreA* probe, the wild-type band was no longer visible in this strain and the *hph* probe still confirmed the homologous integration (Fig. 5E). This strain was recomplemented as described in Materials and Methods (Fig. 5C). As a final proof for the deletion of the *lreA* gene, real-time reverse transcription-PCR (RT-PCR) was performed also to identify small amounts of wild-type mRNA in the deletion strain. In comparison to wild-type results, *lreA* mRNA could no longer be detected. In contrast, the nonpurified heterokaryotic transformant showed a reduction in the expression level. Expression analysis of the recomplemented strain showed *lreA* expression like that of the wild type (Fig. 6F).

Spore formation in blue light is restored in the *lreA* mutant and increased in the dark. With confirmed deletion mutant no. 4 and the recomplemented strain, we analyzed the spore numbers in darkness and blue light in comparison to wild-type numbers. Given that spore formation is inhibited by blue light and that LreA is likely to be involved in blue-light signaling, we expected a release of the repression in the *lreA* mutant. Interestingly, after 7 days of culturing, the amount of spores in the *lreA* mutant strain grown under blue light was slightly higher than in the cultures kept in the dark. Surprisingly, the number of spores obtained in the dark in the *lreA* mutant was 1.61 higher than the number seen with the wild type. The recomplemented strain behaved like the wild type (Fig. 6). These results suggest that LreA acts as a repressor independently of light conditions. Evidence for LreA as a light sensor and activator came from the study of AOH formation.

Blue-light induction of AOH depends partially on LreA. The qualitative detection of the mycotoxins in the TLC assay already

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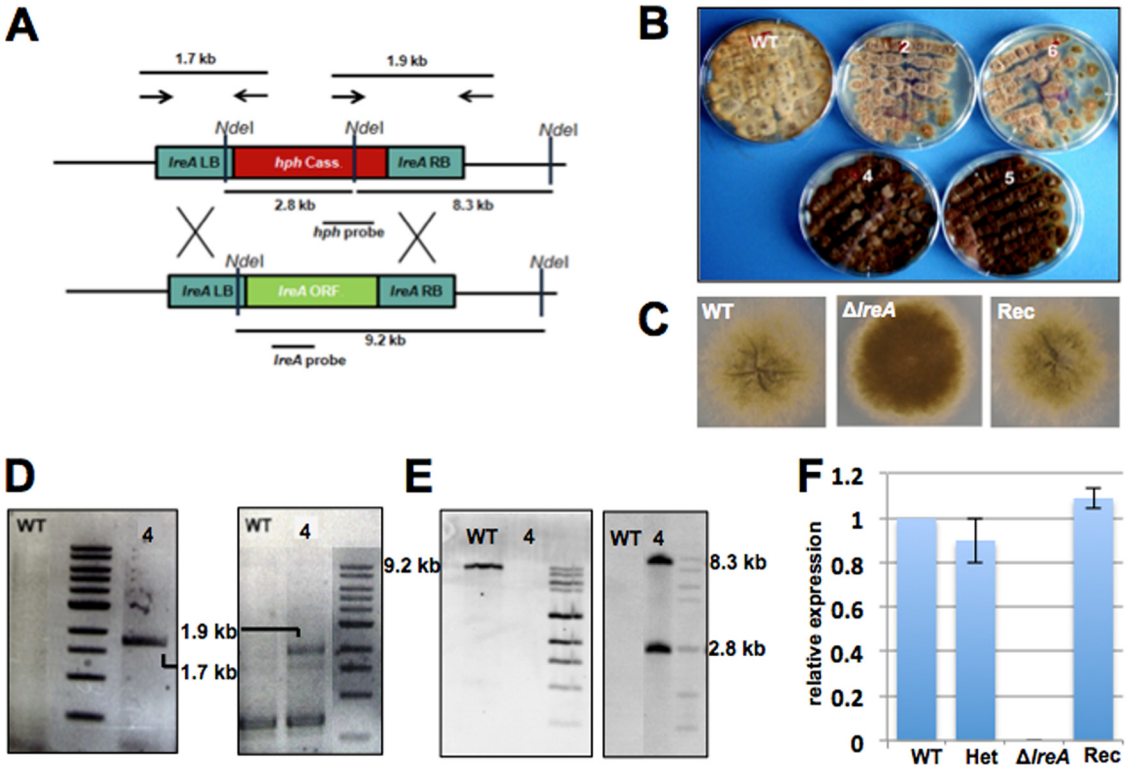

FIG 5 Deletion of *lreA*. (A) Scheme for the homologous recombination of the knockout construct containing *lreA* left and right borders and a hygromycin B resistance cassette (*hph*) at the *lreA* locus. NdeI restriction sites used for the Southern blot are shown. The arrows in the upper part mark the sites of the primers and the sizes of the amplicons shown in panel D. (B) Phenotype of the wild-type (WT) strain and of 4 strains after the last purification step. Only no. 4 and 5 show a clear difference in comparison to the WT. (C) Colony pictures of the wild-type DSM 12633 strain, the recomplementation (Rec) strain, and the knockout strain of *lreA*. Colonies were grown 4 days on solid mCDB media. (D) Confirmation of homologous integration of the deletion construct by PCR with primers binding in the *hph* cassette and upstream or downstream of the regions used for the construct. (Left panel) Amplicon for the upstream region (1.7 kb). (Right panel) Amplicon for the downstream region (1.9 kb). As a marker, a 1-kb ladder (NEB) was used. (E) Southern blot analysis with a probe for *lreA* (left panel) confirms the deletion event. The correct integration is shown with a probe for *hph* (right panel). For the band pattern, see the scheme in panel A. (F) Expression analysis of *lreA* in the wild-type (WT) strain, the heterokaryotic (Het) strain, and the recomplemented (Rec) strain. Strains were incubated 2 days in static liquid culture at 28°C. Expression data of all strains were normalized to wild-type expression.

indicated changes in the concentrations of mycotoxins in *lreA* deletion strains grown in blue-light cultures in comparison to the wild type (data not shown). The increase of the production of AOH in blue light was reduced to only a small increase in the mutant, suggesting an activating function for LreA. In comparison, the wild type produced ATX-I only under blue-light conditions. Interestingly, the *lreA* mutant also produced this compound in the dark, suggesting a repressing function of LreA. To quantify these results, LC/MS analysis has been done with the same samples (Fig. 7A). The results for AOH in the wild type showed a 2.8-fold increase in blue light (Fig. 7B). In contrast, AOH in the *lreA* mutant was increased only slightly by a factor of 1.4 in blue light in relation to the dark. This residual blue-light induction suggests the presence of an alternative blue-light receptor, possibly the cryptochrome (Fig. 1).

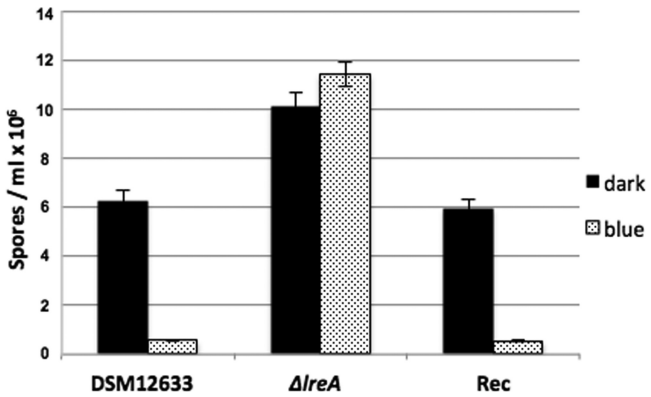

FIG 6 Spore numbers in the *A. alternata* wild-type (WT) strain, the *lreA* knockout strain, and the recomplementation strain. Spores were harvested from the surface of an agar plate after 7 days of incubation either in blue light or in darkness. Three independent plates of each strain were analyzed, and the mean values for the three samples are displayed. The standard deviation is indicated.

Altertoxins I and II were not detectable in the dark in wild-type cultures. After blue-light exposure, ATX-I and ATX-II appeared. In the *lreA* mutant, the concentrations for ATX-I and -II were quite similar in blue light and darkness, with only a slight increase in blue light.

LreA controls the expression of several polyketide synthases. To test the influence of LreA on the expression level of genes involved in secondary metabolism, the expression of several polyketide synthases was measured in the wild-type strain in comparison to the Δ*lreA* mutant in darkness and white light (Fig. 8). Among the 10 polyketide synthase-encoding genes, 2 were silent and only 8 were expressed (7). From those, we have chosen *pksA*,

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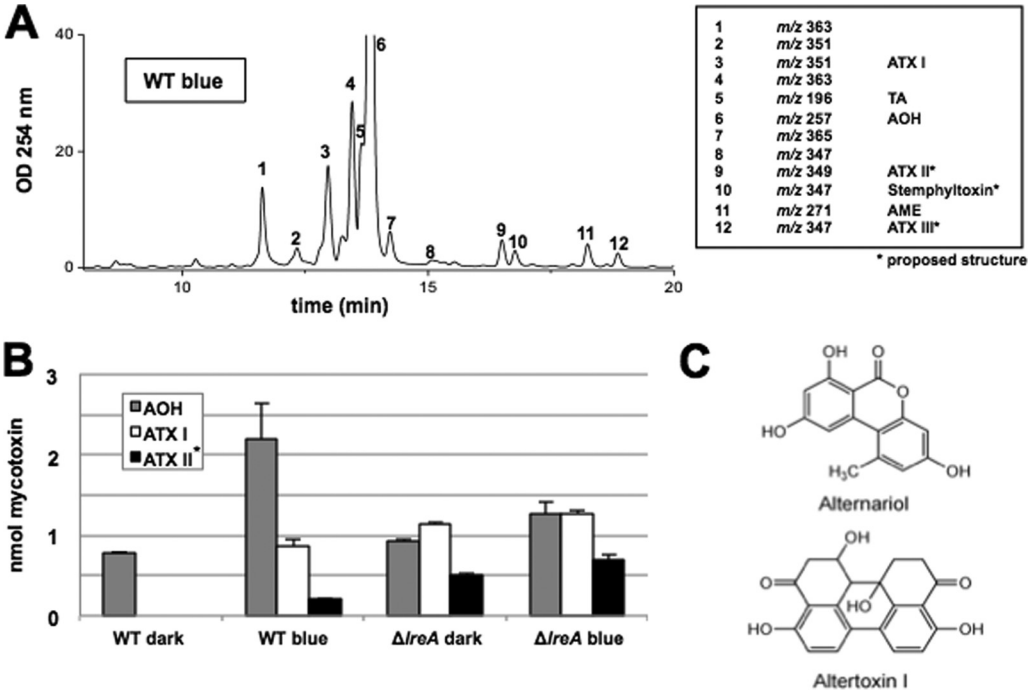

FIG 7 LC/MS analysis of the secondary metabolite profile of the *A. alternata* wild-type (WT) strain and the *lreA* knockout strain. (A) Chromatogram of secondary metabolites extracted from the WT strain grown under blue-light conditions. *m/z* values are given for the most prominent peaks. Known and proposed structures are indicated in the legend. The peak for AOH exceeds the limit of the graph. (B) Secondary metabolite amounts in dark- and blue-light cultures in the WT and *lreA* knockout strains (in nmol/sample). The mycelial dry weights of cultures grown under conditions of blue light and of dark were similar. (C) Formula for alternariol and altertoxin I.

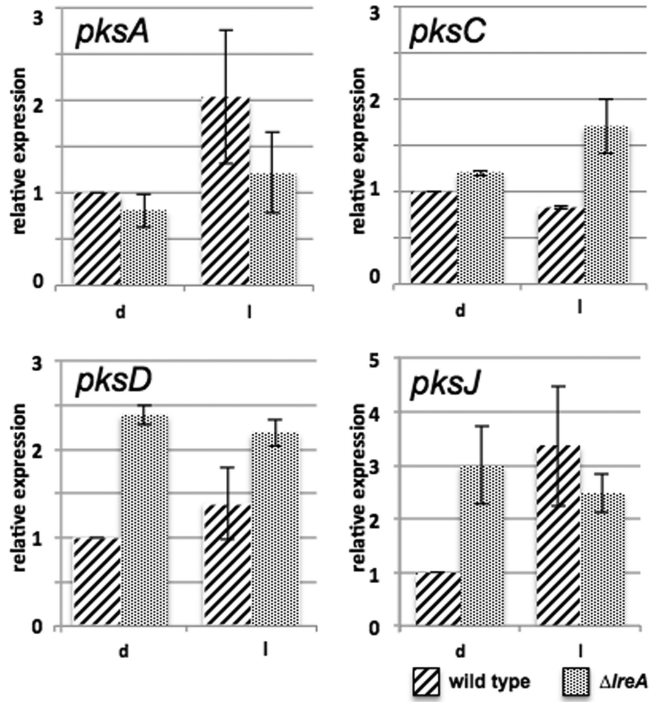

FIG 8 Expression analysis of selected polyketide synthases. Expression of *pksA*, *pksC*, *pksD*, and *pksJ* under dark and light conditions by the wild-type strain is compared to that of the Δ*lreA* mutant strain. All data are normalized to the wild-type dark situation.

because it is involved in melanin biosynthesis (29; R. Fetzner, M. Wenderoth, K. Seither, A. Herr, R. Fischer, submitted for publication), and *pksJ*, because it is involved in alternariol biosynthesis (7). In addition, two genes of unknown function, *pksC* and *pksD*, were chosen. *pksA* was upregulated 2-fold in the wild type under light conditions. This stimulation did not occur in the Δ*lreA* mutant strain, suggesting an activating function of LreA. The levels of expression of *pksC* were similar in dark and light for the wild type but higher in the Δ*lreA* mutant in light, suggesting an inhibiting effect of LreA on the expression of *pksC* in light (Fig. 8). In the wild type, the expression of *pksD* was slightly (1.3-fold) upregulated under light conditions. In the Δ*lreA* strain, in contrast, the expression was upregulated about 2-fold in the dark as well as in light, which suggests an inhibiting effect of LreA independent of light. The expression of *pksJ* was 3.4-fold upregulated in light in comparison to the dark. In the deletion strain, the expression levels were similar in the two light situations. This indicates an inhibiting effect of LreA in darkness. This fits to the data obtained for alternariol formation, where the production is stimulated in the dark in the absence of LreA (Fig. 7B). In summary, our data suggest different roles of LreA at the promoters of different *pks* genes.

DISCUSSION

A. alternata is one of the most common fungal species and normally grows saprophytically. However, a number of different pathogenic variants have been described which produce certain plant-specific toxins. These variants cause necrotic disease on different plants (9, 30). Despite the wide distribution and the great economic impact, the number of molecular studies is rather limited. Only some genes have been analyzed by gene deletion; e.g.,

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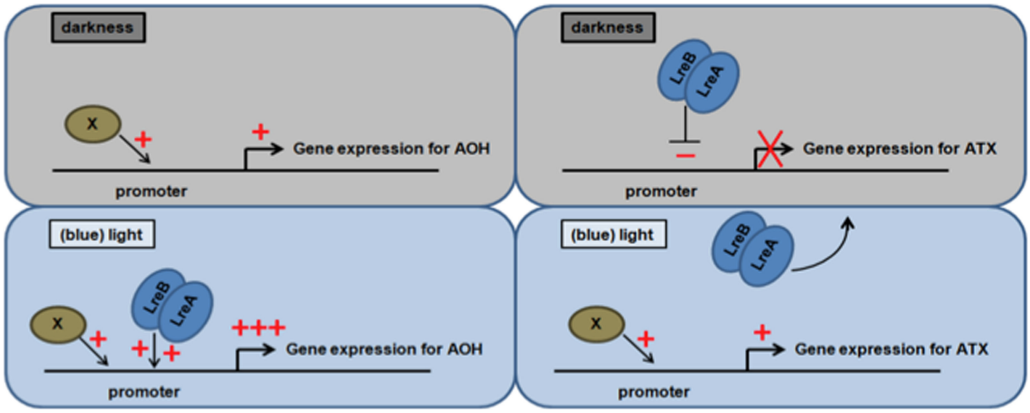

FIG 9 Working model for the LreA-dependent regulation of mycotoxin formation. Expression for AOH and ATX genes is differently regulated by LreA. In the case of AOH, gene expression takes place also in the dark, positively regulated by an unknown factor (left, upper panel). In light, LreA appears to additionally stimulate the expression and the transcription level is increased (left, lower panel). It is unknown whether the effect of LreA is direct or indirect. In contrast, LreA appears to repress the expression of the ATX genes. A complex of LreA and LreB as a transcriptional regulator is proposed in analogy to the situation in *N. crassa* and *A. nidulans*.

the role of peroxisomes in toxin formation has recently been addressed by deletion of a gene encoding a Pex6 homologue (31). In addition to methods for gene deletion by homologous replacement, high-throughput gene disruption methods have been established (20). Another possibility for gene inactivation is the use of RNA silencing, which has recently also been used in *A. alternata* (32). We encountered extreme difficulties in our efforts to obtain a clean *lreA* deletion strain. Apparently, heterokaryon formation is very common and a very stable situation. Clonal purification of strains is hindered by the fact that the asexual spores consist of several compartments, although the nuclear distribution in hyphae and during spore formation suggested earlier that spores are homokaryotic (33). We were unable to obtain homokaryotic strains by plating spores but had to generate protoplasts instead, rather suggesting that spores are heterokaryotic (Fig. 2, bottom panel, inset). Another big problem in the analysis of strains and their phenotypes is the large variability of *A. alternata*. Sometimes the color of the mycelium changes from green to brown or even orange. This might be due to epigenetic modifications or instabilities of the genome. There are several studies that describe a high level of genetic diversity in *A. alternata* (34–36) despite the fact that such great variations are more common for fungi with sexual development and that a sexual cycle for *Alternaria* spp. is not known. One indication for such genomic variabilities is the fact that *A. alternata* harbors a dispensable chromosome, on which the ACT toxin biosynthesis gene clusters are located (32). These properties of *A. alternata* may also explain some apparently contradictory results obtained in our study in comparison to work published earlier by Häggblom (8, 37). However, it has to be considered that the production of secondary metabolites is controlled by many parameters and is also linked to the primary metabolism. It has been reported recently that in *A. nidulans*, changes in the glucose concentration may have drastic effects on the light response (38). Häggblom performed drop culture experiments at a temperature of 20°C. We normally used 28°C because of the optimal growth of *A. alternata* at that temperature. Of course, we performed TLC experiments also with cultures grown at 20°C, but there was no difference from the results at 28°C. Häggblom also used another kind of light source in his blue-light experiments whose intensity (0.21 W/m^2) was lower than that of the light source that we used and kept the dark controls wrapped in aluminum foil, which does often increase the water activity values. The water activity is one of the parameters that is able to influence mycotoxin production. For AOH, a maximum of production at an a_w value of 0.98 has been shown in other studies (39, 40). Given the complexity of the regulation and the as yet rather limited molecular insights into the regulatory networks, it is currently difficult to develop guidelines for food or feed storage to prevent toxin formation by *Alternaria* species. Much more work is clearly necessary to fully understand the production of secondary metabolites.

Another very interesting result of this study is that the white-collar 1 homologue LreA has activating and repressing functions in *A. alternata* (Fig. 9). White-collar 1 has been studied very well in several fungi, and the molecular function is best known in the control of the circadian clock in *N. crassa* (41–44). Here, WC-1 forms a complex with WC-2 (WCC) which acts, for instance, as a positive regulator for the negative-feedback protein Frequency and the photoadaption protein Vivid (44, 45). It is light activated and directly binds to the promoters of light-regulated genes (46). We found that *A. alternata* LreA apparently fulfills a repressing function in the dark as well as an activating function in light in the control of secondary metabolism and sporulation. Interestingly, mycotoxin production is not coupled with sporulation, which was described for several mycotoxigenic genera, including *Aspergillus* (47), *Claviceps*, *Penicillium*, and *Fusarium* (26). In *A. alternata*, the highest toxin levels were obtained in blue light, where sporulation in contrast is strongly inhibited. The repressing function of a white-collar homologue has been reported before for *A. nidulans*, although the derepressing effect on conidiospore formation was very small (Purschwitz et al. [16]).

Although our results do not allow us to speculate further at the moment on a molecular model explaining how this repressing effect can be mediated and on the effect of light, the action as a repressor of the ATX-I and ATX-II biosyntheses is especially interesting because LreA might also repress other secondary metabolite biosyntheses. A complete profiling of secondary metabolites in an *A. alternata* wild type and the *lreA* mutant may reveal novel

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secondary metabolites which are normally not produced under laboratory conditions. Recent studies, especially in *A. nidulans*, revealed that the induction of sleeping gene clusters is a potent strategy for the discovery of novel, bioactive secondary metabolites (48–50).

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