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DISSERTATION

The Role of Methyltransferase LAE1 and Related Proteins for *Trichoderma* Interaction with its Environment

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In Zusammenarbeit mit

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Research is what I'm doing when I don't know what I'm doing

Wernher von Braun

I lovingly dedicate this thesis to my parents, who supported me each step of the way

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ABSTRACT

In *Aspergillus* spp. the *laeA* (loss of *aflR* expression) gene encodes a putative methyltransferase that acts in a complex with VeA, i.e., Velvet A, and VelB i.e., Velvet-like B, as master regulator of secondary metabolism, virulence as well as development. LaeA is also required for the biosynthesis of secondary metabolites in the penicillin producer *Penicillium chrysogenum* and the phytopathogenic fungi *Fusarium fujikuroi* (e.g., gibberellins) and *Cochliobolus heterostrophus* respectively- In all these processes, LaeA acts at the level of chromatin modification thereby rendering gene clusters to be coordinately transcribed. Reports about the involvment of LaeA in other cellular processes is not available.

Trichoderma is a genus of filamentous fungi with broad range of applications. *T. reesei* is well known for its production of enzymes used for plant biomass hydrolysis whereas *T. atroviride* is conspicuous for its vigorous mycoparasitism and is therefore industrially applied as a biocontrol agent. The genes encoding cellulases are found in clusters in genome and expressed coordinately. Similarly, genomic clustering of mycoparasitic genes have been also observed in *T. atroviride.*

In order to learn whether LAE1 of *T. reesei* is involved in the expression of cellulase genes I used reverse genetics to show that is indeed the case: in a *lae1* deletion mutant we observed a complete loss of expression of all seven cellulases, auxiliary factors for cellulose degradation, β-glucosidases and xylanases were no longer expressed. Conversely, enhanced expression of *lae1* resulted in significantly increased cellulase gene transcription. Interestingly, chromatin immunoprecipitation followed by highthroughput sequencing ("ChIP-seq") showed that *lae1* expression was not obviously correlated with H3K4 di or trimethylation (indicative of active transcription) or H3K9 trimethylation (typical for heterochromatin regions) in CAZyme coding regions. Genome-wide ChIP sequencing detected 4089 genes bearing one or more of these methylation marks, of which 75 exhibited a correlation between either H3K4me2 or H3K4me3 and regulation by LAE1, suggesting that LAE1 does not directly modulate H3K4 or H3K9 methylation. Genome-wide gene expression analysis showed that *lae1* positively regulates 7 of 17 polyketide or nonribosomal peptide synthases, genes encoding ankyrin-containing proteins, iron uptake, heterokaryon incompatibility proteins, PTH11-receptors, and oxidases/monoxygenases are major gene categories also regulated by LAE1. To investigate whether the Velvet A protein would be required for the regulation of cellulase formation by LAE1, I characterized the *T. reesei* orthologue *vel1*. Deletion of *vel1* did not affect cellulase gene expression in *T. reesei*, but *vel1* overexpression strongly enhanced it. Similar findings were also obtained for the formation of xylanase and ß-xylosidase enzyme activities. The stimulation of cellulase gene expression by overexpressing *vel1* was dependent on a functional *lae1* allele, suggesting the operation of a *vel1*-independent pathway of activation by LAE1 in *T. reesei*.

In *T. atroviride*, loss of function of *lae1* results in a loss of mycoparasitic activity correlated with a significant underexpression of several genes normally upregulated during mycoparasitic interaction (proteases, GH16 ß-glucanases, polyketide synthases and small cystein-rich secreted proteins), which was also reflected in the partial reduction of formation of fungicidal water soluble metabolites and volatile compounds. Summarizing, my data point to new roles of LAE1 in the physiology of *Trichoderma*. LAE1 appears to be a regulator of the fungus fitness in its specialized habitat.

ZUSAMMENFASSUNG

Das zuerst in *Aspergillus* spp. gefundene *lae1* -Gen kodiert für eine putative Methyltransferase, die in einem Komplex mit VeA,(VeA und VELB)als Master-Regulator des Sekundärstoffwechsels, der Virulenz sowie der Entwicklung wirkt. LaeA reguliert auch die Biosynthese von Sekundärmetaboliten in dem Penicillin-Produzenten *Penicillium chrysogenum* und den phytopathogenen Pilzen *Fusarium fujikuroi* (zB Gibberelline) und *Cochliobolus heterostrophus*. In allen diesen Prozessen wirkt LaeA auf Ebene der Chromatin Modifikation, und ermöglicht dadurch die koordinierte Transkruiption von Genclustern. Berichte über die Beteiligung von LaeA in anderen zellulären Prozessen sind nicht verfügbar.

Trichoderma ist eine PIlzgattung mit breiten Anwendungen. *T. reesei* ist bekannt für seine Produktion von Enzymen für die Hydrolyse pflanzlicher Biomasse, während *T. atroviride* sich durch einen ausgeprägten Mykoparasitismus aufweist und daher industriell als Biokontrollmittel angewandt wird. Die Gene, die Cellulasen kodieren, befinden sich in Clustern in Genom und werden koordiniert exprimiert. Ein genomisches Clustering von unter mykoparasitischen Bedingungen exprimierten Genen ist auch in *T. atroviride* beobachtet worden.

Um herauszufinden, ob LAE1 von *T. reesei* die Expression von Cellulasen reguliert verwendete ich einen reversen genetischen Ansatz: in einer *lae1* Deletionsmutante beobachtete ich einen vollständigen Verlust der Expression aller sieben Cellulasen, Hilfs-Faktoren für die Celluloseabbau, β-Glucosidasen und Xylanasen. Eine deutlich erhöhte Cellulasetranskription ergab sich in Mutanten mit erhöhter Expression von LAE1. Chromatinimmunpräzipitation, verbunden mit Hochdurchsatz-Sequenzierung ("ChIP-seq") zeigte jedoch, dass offensichtlich die LAE1 Expression nicht mit H3K4 di oder Trimethylierung (indikativ aktiver Transkription) oder H3K9 Trimethylierung (typisch für Heterochromatin Regionen) in CAZyme kodierenden Regionen korreliert. Genom-weite ChIP-Sequenzierung entdeckte 4089 Gene, die eine oder mehrere dieser Methylierung Marken ertragen, von denen 75 zeigten eine Korrelation zwischen beiden H3K4me2 oder H3K4me3 und Regulierung durch LAE1, was darauf hindeutet, dass LAE1 nicht direkt H3K4 oder H3K9 Methylierung moduliert.

Eine genomweite Genexpressionsanalyse zeigte ferner, dass LAE1 positiv die Transkription von 7 der 17 Polyketid- oder nicht-ribosomalen Peptidsynthetasen reguliert. Außerdem wurden Gene, Proteine mit Ankyrin-Domainen, Eisenaufnahme, Heterokaryon Inkompatibilitäts-Proteine, PTH11-Rezeptoren und verschiedene Oxidasen / Monoxygenasen kodieren, durch LAE1 reguliert. Um zu untersuchen, ob das Velvet A Protein aus *T. reesei* für die Regulierung der Bildung von Cellulase durch LAE1 erforderlich ist, wurde das *T. reesei* Ortholog von *vel1* charakterisiert. Deletion von *vel1* beeinflusste nicht die Cellulase Genexpression in *T. reesei*, aber *vel1* Überexpression hat sie stark verbessert. Ähnliche Ergebnisse wurden auch für die Bildung von Xylanase und ß-Xylosidase Enzymaktivitäten erhalten. Die Stimulierung der Cellulase Genexpression durch Überexpression von *vel1* war abhängig von einer funktionellen LAE1 Allel und damit wurde ein *vel1*-unabhängigen Modus der Aktivierung durch LAE1 in *T. reesei* angedeutet.

In *T. atroviride* führte der Verlust der Funktion von LAE1 zu einer stark verringerten mykoparasitischen Aktivität und einer signifikanten Unterexpression von mehreren Genen, die früher als unter mykoparasitischer Interaktion hochreguliert gezeigt wurden, wie z. B Proteasen, GH16 ß-Glucanasen, Polyketidsynthasen und kleine Cystein-reiche sekretierte Proteine. Dies korrelierte auch mit der teilweisen Verringerung der Bildung von fungiziden wasserlöslichen Metaboliten und flüchtigen Metaboliten. Zusammenfassend deuten meine Daten auf neue Rollen von LAE1 in der Physiologie von *Trichoderma* hin. LAE1 scheint ein Regulator des Fitness von Pilzen in ihrem spezifischen Habitat zu sein.

CONTENTS

Chapter 1

General Introduction

Filamentous fungi as cell factories of natural chemicals

Fungi are members of a large group of eukaryotic organisms that recently have gained extensive attentions in medicine, agriculture and industry. Production of recombinant proteins by using filamentous fungi as hosts of recombinant protein products have been the subject of massive studies in over past decades. These organisms are dominant producers of a range of primary metabolites such as citric acid, microbial lipids (biodiesel) and higher unsaturated fatty acids (HUFAs). In particular, they produce also important secondary metabolites with enormous therapeutic applications in biopharmaceutical industry, for example: cephalosporin, penicillin, taxol, zeranol and ergot alkaloids (Ward 2012). Notwithstanding, recent advances in molecular genetic tools have enabled us to use fungi for expressing both endogenous and exogenous genes, but still application of these organisms as hosts for production of recombinant proteins is limited mainly due to their common ability to produce proteases which can degrade the heterologous protein. On the other hand, it is important to increase the expression and secretion of desired product in fungi, therefore yet ongoing studies in multidisciplinary fields such as engineering, molecular genetics and microbiology are required. Such studies should direct us to exploitation of new bioprocess strategies as well as improving gene expression systems in filamentous fungal hosts and to find alternative new species by exploring new niches and geographical locations and thereby to inhibit protease activity, understand molecular mechanisms of transcription regulation in these organisms and ultimately to improve and identify better fungal producer strains and species (Punt et al. 2002, Ward 2012, Nevalainen et al. 2005, Wang et al. 2005).

Trichoderma: A fungal genus as producer of lignocelluloses degrading enzymes and biocontrol agents

Trichoderma is a genus of holomorphic fungi that was proposed first by Persoon in 1794. Most of *Trichoderma* species can also propagate through sexual life cycle rather than asexual reproductive style via spores. Teleomorphs of *Trichoderma* are species of the ascomycete genus *Hypocrea*. *Trichoderma spp*. have been demonstrated as efficient producer of highly active cellulolytic enzymes (Kubicek and Harman 1998). This trait make them effective in disrupting and depolymerization of lignocellulosic materials and thus applicable tools in number of biotechnological areas as diverse as clothes-washing detergent, animal feed, pulp and fuel productions.

Genome sequencing data of several *Trichoderma* species are available (http://genome.jgi.doe.gov/). Martinez et al (2008) reported co-clustering of carbohydrate active enzyme genes (CAZyme) in genome of *Trichoderma reesei* -the fungus regarded as the main industrial progenitor and source of cellulaes and hemicellulases. Moreover, these authors showed that, this fungus contains less number of genes encoding CAZymes when compared to other ascomycete fungi such as *Podospora anserina, Gibberella zeae* or *Magnaporthe grisea* and that some of these clusters are found in regions in vicinity of either non-ribosomal peptide synthetases (NRPS) or polyketide synthases (PKS). Recently, the CAZyme gene content of *T. reesei* was updated and reannotated trough computational and manual approaches (Häkkinen et al. 2012). In this study, 201 gylcoside hydrolase genes, 22 carbohydrate esterase and five polysaccharide lyase genes were identified. Moreover, GH3-β-glucosidases, GH18 chitinases and GH27 α-galactosidases showed functional diversity within analyzed CAZyme genes.

The control of plant pathogens for plant protection by interference with their ecological status, as by introducing a natural enemy or a pathogen into the environment is called “biocontrol”. Due to their ubiquitous occurrence in root ecosystems and interactions with other fungi, animals and plants, *Trichoderma* spp gained the ability for having remarkable various lifestyles ranging from being

opportunistic, endophytic, saprophytic and avirulent plant symbionts to parasites of other fungi (Druzhinina et al. 2011) and thus are used as biocontrol agents. Mycotrophy is considered as direct interaction of *Trichoderma* (predator) with other fungi (prey) that results in efficacious feeding on either living or dead fungal biomass via various events. A combination of several traits such as host recognition, attachment to and sometimes coiling around the host hyphae as well as secretion of antibiotic metabolites (secondary metabolites such as gliotoxin, peptaibols, 6-pentyl-2H-pyran-2-one [6PP]) and cell-wall-degrading enzymes (hydrolytic enzymes including chitinases, glucanases and proteases) and of heterotrimeric G proteins and their receptors in sensing of host signals are involved in this lifestyle (Druzhinina et al. 2011, Harman et al. 2004). In support of this, comparative genome sequencing analysis of the two biocontrol species, *T. atroviride and T. virens*, with *T. reesei* and ten additional ascomycete, whose genomes are available in the Joint Genome Institute (JGI), revealed that several mycoparasitism-specific gene families such as enzymes involved in chitin degradation (family GH18 of CAZymes, chitinases contain carbohydrate binding domains and GH75 chitosanases) and proteases are expanded in *T. atroviride* and *T. virens* relative to *T. reesei* and other ascomycetes. Although - except *T. virens*- genes encoding secondary metabolites (PKS, NRPS and PKS-NRPS) are not significantly more expanded than in some other fungi; however are yet in *T. atroviride* more abundant than *T. reesei* (Kubicek et al. 2011).

LaeA as global regulator of secondary metabolism

LaeA was originally identified as a nuclear protein that regulates multiple secondary metabolite gene clusters in *Aspergillus* (Bok and Keller 2004, Keller et al. 2006). Deletion of *laeA* in *A. fumigatus* resulted in impaired production of several toxins such as gliotoxin, fumagilin, fumagatin and helvolic acid (Bok et al. 2005). Moreover, loss of function of this protein in *A. nidulans, A. flavus and A. terreus* blocked strigmatocystin (ST), penicillin (PN) and lovastatin (LOV) gene expression in these fungi (Bok and Keller 2004). Later on, this protein was also characterized in other fungi, including

Fusarium fujikuroi, *Penicillium chrysogenum* and *Cochliobolus heterostrophus* (Wiemann et al. 2011, Kosalková et al. 2009, Wu et al. 2012). In *F. fujikuroi* - a fungus which besides its role as industrial producer of gibberellins (GAs), can also produce additional secondary metabolites (SMs) such as neurosporaxanthin, bikaverin, fumonisins and fusarin C- LAE1 plays a role as activator of GA biosynthesis (Wiemann et al. 2011). In addition, cross genus complementation studies showed that F. *fujikuroi lae1*, *P. chrysogenum laeA* and *A. nidulans laeA* are functionally conserved proteins among these fungi (Wiemann et al. 2011). The same findings were obtained in *Chlae1* knock-out mutant of *C. heterostrophus* – the maize pathogen fungus known to produce T-toxin. T-toxin production and *Tox1* gene expression were minimal in *Chlae1* mutant whereas increased significantly in *Chlae1* overexpression strain compared to wild type (Wu et al. 2012). Similarly, production of penicillin is reduced drastically in industrial fungus, *P. chrysogenum*, in *PclaeA* knock-down mutants, but, expression of the *dmaW* involved in roquefortine biosynthesis and the levels of roquefortine C remained similar to those observed in the wild-type (Kosalková et al. 2009).

The virulence of human pathogenic fungus, *A. fumigatus*, was decreased drastically by deletion of *laeA*. *AflaeA* mutants were impaired in killing polymorphonuclear neutrophils cells and showed decreased fatal infections in the murine model (Bok et al. 2005). Reduced degree of pathogenicity of *F. fujikuroi* during infection of rice seedlings with *F. fujikuroi lae1* knock-out strains is most likely due to its attenuated capacity of the strains for formation of gibberellins influencing various developmental processes in plant growth (Wiemann et al. 2011). Strains lacking *lae1* in *C. heterostrophus* have shown significant reduction of lesion sizes on normal cytoplasm (N-cytoplasm) leaves while in contrast, overexpressing mutants produced smaller lesions than parent strain and consequently lower and higher amounts of T-toxin respectively (Wu et al. 2012). Taken together, these data suggest that LaeA is a virulence factor in all pathogenic fungi examined to date.

Velvet complex, a multicomponent regulator of secondary metabolites production and morphogenesis

Secondary metabolism and differentiation are tightly-associated processes influenced by light. Heterotrimeric velvet complex comprising VelB/VeA/LaeA was identified as a coordinator connecting light-dependent developmental regulation and biosynthesis of secondary metabolites (Bayram et al. 2008). In well-studied model *A. nidulans*, LaeA as a global regulator of SM metabolism is bridged via VeA to VelB in dark: Disappearance of light increases the transport of VeA from cytoplasm to nucleus while localization of VelB remains almost unaffected by illumination. On the other hand, constitutive-nucleus-localization of LaeA establishes the interaction of VeA and LaeA in nucleus for which the presence of VelB is also required thus forces that VelB to enter to nucleus (Bayram et al. 2008). The interplay between secondary metabolism, light and development is also described by aforementioned interaction between components of velvet proteins. Evidence of connection between sexual lifestyle (formation of fruiting bodies), light and secondary metabolism has been proved already in studies performed in genetically amenable organism *A. nidulans* (Kim et al. 2002, Busch et al. 2003). In this organism, sexual reproduction and secondary metabolite production are inhibited by presence of light. Deletion of two members of velvet complex, VeA and LaeA, resulted in mutants impaired in secondary metabolism and formed smaller fruiting bodies which is attributed to the lack of specific globose (or "Hülle") cells, which nurse the young fruiting body during development (Calvo et al. 2004, Wu et al. 2012, Bayram et al. 2010). In addition to the formation of sexual recombination, also asexual reproduction (formation of conidia and chlamydospores), as well as formation of sclerotia is also under control of components of velvet (Cary et al. 2007, Amaike et al. 2009, Schumacher et al. 2012., Jiang et al. 2011, Kopke et al. 2012, Hoff et al. 2010, Mukherjee and Kenerley 2010, Wiemann et al. 2011). Growth of aerial hyphae of *C. heterostrophus* was decreased in *Δlae1* mutant while asexual sporulation was increased, additionally, deletion of LAE1 and Vel1 in this fungus resulted in female sterility (Wu et al., 2012). Loss and increase of sclerotia formation occurred

as a result of deletion and overexpression of *laeA* allele in *Aspergillus flavus* (Kale et al., 2008). Altered conidial development including reduced conidial chain elongation and colony hydrophobicity, but, adversely increased production of conidiophores was observed in *A. flavus* CA14 *laeA* deletion mutant when compared to wild type strain (Chang et al., 2012). P*claeA* knock-down mutants showed defective pigmentation and sporulation as compared to *P. chrysogenum* WT strain (Kosalková et al., 2009, Hoff et al., 2010). In addition, morphogenesis (branching of hyphae, hyphal swelling and aerial mycelia formation) are also affected by manipulation of velvet proteins (Choi and Goodwin, 2010).

How does LaeA work?

Chromatin-based control is involved in several silencing phenomena observed in fungi such as silencing of ribosomal DNA, telomere position effect and regulation of genes involved in nutrient acquisition. These phenomena often take place near centromeres and telomeres, where facultative heterochromatin displays a role in transcription. There are co-regulated gene clusters of secondary metabolites in fungi which are often found in sub-telomeric regions of genome. Moreover, the presence of a conserved S-adenosyl-L-methionine (SAM) binding domain in LaeA (Bok et al. 2004), and nuclear localization of this protein, have been shown already by different workers (McDonagh et al. 2008, Perrin et al. 2007). On the other hand, LaeA governs transcriptional control of about 10% of the genes contained in the *A. fumigatus* genome (Perrin et al. 2007), including a 70 kb area containing the sterigmatocystin cluster in *A. nidulans* (Bok and Keller et al. 2004). Taken together, these studies suggest that LaeA is an S-adenosylmethionine-dependent (SAM) protein methyltransferase that acts at the level of chromatin remodeling in regulation of secondary metabolism. To gain insight into the mechanism of function of *laeA*, the effect of histone modifying genes [(e.g., HdaA (a histone deacetylase (HDAC), ClrD (H3K9 methyltransferase) and HepA (heterochromatin protein 1), who activate or silence, respectively, gene clusters for secondary metabolite synthesis)] on phenotypes arising in LaeA mutants has been investigated.

The involvement of chromatin in regulation of secondary metabolites biosynthesis was suggested by the findings on involvement of HDACs in the regulation of synthesis of several secondary metabolites in *Aspergillous* (Lee et al. 2009, Shwab et al. 2007). Deletion of *hdaA* in *A. nidulans* caused transcriptional activation of two telomere-proximal gene clusters, i.e. ST and PN, which was correlated by an increased biosynthesis of the respective products. In addition to this, treatment of this fungus with HDAC inhibitors led to elevated expression of aforementioned metabolites, the similar observation which could be extended also to unrelated species such as *A. alternata* and *P. expansum* and thus suggesting a conserved mechanism of HDAC in activation of SMs genes through acetylation of chromatin and thereby establishing euchromatin (Shwab et al. 2007). Chromatin immunoprecipitation (ChIP) of *Aspergillus parasiticus* genome with histone-K4ac showed enrichment of histone H4 acetylation in aflatoxin promoters and thus concomitantly accumulation of aflatoxin and aflatoxin protein (Roze et al. 2007). Noteworthy to mention, studies on *A. parasiticus* showed that other elements such as cAMP response element binding protein (CRE1bp) and histone acetyltransferase (HAT) are also postulated to mediate this process [(Roze et al. 2004b, c.f (Shahbazian and Grunstein 2007)].

Reyes-Dominguez et al. (2010), reported that in *A. nidulans*, deletion of HepA and ClrD increase ST biosynthesis which was accompanied by decreased tri-methylation of H3K9 inside ST cluster. Double knock-out mutant of *hepA*/*laeA* showed partial remediation of *aflR* expression in comparison with *ΔlaeA* strain which was also the case in *ΔClrDΔlaeA* strain only after 5 days incubation on solid GMM (glucose minimal medium) thus implying that LaeA counteracts formation of heterochromatin mediated by HepA/ClrD complex.

As mentioned earlier, this nuclear protein contains seven-beta-strand methyltransferase motifs; however, to date no protein that becomes methylated by LaeA has been identified. Most recently, Panatananan et al. (2013), in the attempt to identify the methyl-accepting substrate of *A. nidulans* LaeA, reported that an automethylation reaction is the major enzymatic reaction of LaeA.

Importantly, neither histones nor the velvet complex proteins were methylated by the putative methyltransferase LaeA (Panatananan et al. 2013).

To summarize, in spite of growing body of evidences which confirm that secondary metabolite regulations are Likely linked to histone modifications, but, yet the questions whether LaeA can change the chromatin structure directly or indirectly and its targets remain open and thus the precise function of that is still enigmatic and await further investigations.

Why *Trichoderma*?

As described earlier, genome analysis of *T. reesei* revealed surprisingly that in spite of its high capacity for digesting cell wall compounds, *T. reesei* contains fewer genes encoding cellulases, hemicellulases and pectinases necessary for depolymerizing plant cell wall polysaccharides (Martinez et al. 2008). However, many CAZyme encoding genes are nonrandomly distributed within the genome; in concordance, several of the regions with high density of CAZyme genes also contain genes encoding proteins involved in SMs biosynthesis. In particular, from 25 CAZyme clusters, five of them are co-located in regions where either a polyketide synthase (PKS) gene or a nonribosomal peptide synthase (NRPS) gene is also present. Co-clustering of CAZyme genes with several SMs clusters, may enable *Trichoderma* to avert competition for nutrients and thus promotes the organism survival and fitness in interaction with its environment by the aid of an arsenal of antibiotic secondary metabolites (Martinez et al. 2008).

Moreover, transcriptional analysis of biomass degrading enzymes of *T. reesei* showed that most of the genes encoding aforementioned enzymes were transcriptionally co-regulated. Intriguing, several of these enzymes are not thought to degrade cellulose directly (Foreman et al. 2003).

Furthermore, comparative genome sequence analysis of the two biocontrol species of *Trichoderma* capable of mycoparasitism (*T. atroviride* and *T. virens* with *T. reesei*) demonstrated an expansion of genes with potential relevance for mycoparasitism (Kubicek et al. 2011). These genes comprised

CAZyme genes encoding enzymes hydrolyzing chitin/chitosan (GH18, GH75 and GH17 families) and β-glucan (GH55, GH64 and GH81 families), which are found in highest amount in *T. virens* and *T. atroviride* among all described fungi in this study (Kubicek et al. 2011). NRPS, PKS and PKS-NRPS fusion genes are typical secondary metabolite genes present abundantly in total numbers equal to 50 and 35 in *T. virens* and *T. atroviride* respectively, whereas this is only 23 in *T. reesei.*

Given these information exploited out of the genome mining of *T. reesei* on co-clustering of cellulase genes, together with in-above-described co-regulation of biomass degrading enzymes, it should be therefore invaluable first to postulate that the function of the orthologous *T. reesei* protein methyltransferase LAE1 can be expanded also to regulation of other as-yet-unreported cellular processes such as cellulase genes expression and second: (if proven) the *T. reesei* VeA ortholog, VEL1 contributes in regulation of cellulase formation by interplaying with LAE1 .On the other hand, an arsenal of various SMs genes as well as clustering of expressed mycoparasitism genes together, in *T. atroviride*, should raise the necessity of investigation of LAE1 influence on mycoparasitism ability of *T. atroviride.* Eventually, this would enable us to examine the possibility of improvement of *Trichoderma* species as producer of value-added enzymes and biocontrol agents via manipulating LAE1 and/or VEL1 and thus likely at chromatin level.

Chapter 2

Scope of this Work

Scope of this work

Based on the available literature, LAE1 is a putative protein methyltransferase unique to filamentous fungi that plays a key role in transcriptional regulation of SMs gene clusters as well as development of fungi. Its role in other cellular processes has so far not been tested. Also based on the available literature, *T. reesei* cellulases are clustered in the genome and coordinately expressed. In addition, there is also a genomic clustering of expressed mycoparasitic genes in *T. atroviride*.

The hypothesis and objective of my work was therefore to investigate the function of LAE1 in *T. reesei* and *T. atroviride*, and specifically its possible involvement in cellulase genes expression. In order to examine the mechanism of regulatory action of *lae1* gene as postulated in *Aspergillus* by chromatin remodeling, also genome-wide chromatin immunoprecipitation followed by high-throughput sequencing ("ChIP-seq") was performed. Further, the involvement of *lae1* gene in the mycoparasitic raection ability of *T. atroviride* to other fungi should be assessed. Finally, I also aimed to address whether the developmental regulator VEL1 is cooperating with LAE1 in its regulation of cellulase gene expression.

This work obviously required a number of international collaborations, in which also some of the experiments that will be described have been performed: I therefore thank Prof. Nancy P. Keller, Dr. Jin Woo Bok and Katherine Rhee for the cross-expression of *lae1* in *Aspergillus nidulans* and the performance of the yeast two-hybrid system analyses; Prof. Michael Freitag, Dr. Kristina Smith and Pallavi A. Phatale for performing the ChIP experiments; and Scott E. Baker for his help in the genomic annotation of *Trichoderma lae1*. At TU Wien, Susanne Zeilinger, Markus Omann, and Alex Lichius helped by performing some of the gene expression analyses related to the characterization of the LAE1-dependent transcriptome and fluorescence microscopy, and to Lea Atanasova and Benigno Aquino for performing some of the work on the characterization of the *vel1* mutants. All other experiments and results described were performed and obtained by myself, under the supervision of

Christian P. Kubicek in collaboration with Bernhard Seiboth (chapters 3 and 4) and Irina S. Druzhinina (chapters 5 and 6).

Chapter 3

The Putative Protein Methyltransferase LAE1 Controls Cellulase Gene Expression in *Trichoderma reesei*

The results presented in this chapter have been published

Bernhard Seiboth, Razieh Karimi Aghcheh, Pallavi A. Phatale, Rita Linke, Dominik G. Sauer, Kristina M. Smith, Scott E. Baker, Michael Freitag and Christian P. Kubicek

LAE1 is essential for cellulase formation in *Trichoderma reesei,* but does not affect CAZyme gene expression by directly modulating H3K4 or H3K9 methylation
Molecular Microbiology (2012) **84**(6), 1150–1164

Abstract

Trichoderma reesei is an industrial producer of enzymes that degrade lignocellulosic polysaccharides to soluble monomers, which can be fermented to biofuels. Here we show that the expression of genes for lignocellulose degradation are controlled by the orthologous *T. reesei* protein methyltransferase LAE1. In a *lae1* deletion mutant we observed a complete loss of expression of all seven cellulases, auxiliary factors for cellulose degradation, β-glucosidases and xylanases were no longer expressed. Conversely, enhanced expression of *lae1* resulted in significantly increased cellulase gene transcription. *lae1*- modulated cellulase gene expression was dependent on the function of the general cellulase regulator XYR1, but also *xyr1* expression was LAE1-dependent. LAE1 was also essential for conidiation of *T. reesei*. Chromatin immunoprecipitation followed by highthroughput sequencing ("ChIP-seq") showed that *lae1* expression was not obviously correlated with H3K4 di or trimethylation (indicative of active transcription) or H3K9 trimethylation (typical for heterochromatin regions) in CAZyme coding regions, suggesting that LAE1 does not affect CAZyme gene expression by directly modulating H3K4 or H3K9 methylation. Our data demonstrate that the putative protein methyltransferase LAE1 is essential for cellulase gene expression in *T. reesei* through mechanisms that remain to be identified.

Introduction

The β-(1,4)-linked glucose polymer cellulose and hemicellulose polysaccharides of varying composition make up 60–80% of the plant cell wall and arise from the utilization of solar energy and carbon dioxide by plants at an annual production rate of about 7.2 and 6 X 10^{10} tons respectively (Gilbert, 2010). These polymers make up a significant portion of the total plant biomass, and degradation of these polysaccharides is a key transformation step in the biological carbon cycle in nature. Most industrial production of enzymes for plant biomass hydrolysis is performed with mutants of the fungus *Trichoderma reesei* (the anamorph of the tropical ascomycete *Hypocrea*

jecorina) (Xu et al., 2009). Consequently, this fungus serves as the model system for the molecular understanding of cellulase gene expression and secretion of the encoded cellulase proteins. To this end, its genome has recently been sequenced (Martinez et al. 2008). Interestingly, *T. reesei* contains a lower number of genes coding for cellulases and hemicellulases when compared with genomes of other ascomycete fungi, such as *Podospora anserina, Gibberella zeae* or *Magnaporthe grisea* (Espagne et al. 2008). Moreover, in contrast to all other ascomycetes whose genome has been sequenced*, T. reesei* cellulase, hemicellulase and other carbohydrateactive enzyme (CAZyme) encoding genes were found to be fivefold enriched in several discrete clusters. Regions of high CAZyme gene density also contain genes encoding secondary metabolic enzymes, such as non-ribosomal polypeptide synthases (NRPS) and polyketide synthases (PKS) (Martinez et al. 2008). Genes encoding enzymes involved in the biosynthesis of secondary metabolites are known to occur in clusters, often near the telomeres of chromosomes (Keller and Hohn, 1997). In the ascomycete genus *Aspergillus*, such clusters of secondary metabolite genes are proposed to be regulated at the level of histones by the putative protein methyltransferase LaeA (Bok and Keller, 2004; Bayram et al. 2008), which somehow reverses gene repression at the level of heterochromatin structure (Reyes-Dominguez et al. 2010; Strauss and Reyes-Dominguez, 2011). Because of the co-clustering of genes for cellulases, hemicellulases and other CAZymes with those for secondary metabolite synthesis in the *T. reesei* genome, we hypothesized that cellulase expression may be regulated by a *T. reesei* LaeA orthologue. Here we describe the identification of a LaeA orthologue in *T. reesei*, LAE1. Furthermore, we show that manipulation of its expression has a dramatic effect on cellulase and hemicellulase gene expression. However, this phenotype is not associated with alterations in H3K4 or H3K9 methylation at cellulase and hemicellulase loci, suggesting that the effect on gene expression is indirect.

Results

Identification of the *T. reesei* LAE1 orthologue

To identify *lae1*, we first screened the 92 predicted *S*-adenosylmethionine-dependent methyltransferases in the *T. reesei* genome database (http://genome.jgi-psf.org/Trire2/Trire2.home.html). When any of the functionally verified *Aspergillus* LaeA proteins (Bok et al. 2005; Bayram et al. 2008) were used as a query in BLASTP, PSI-BLAST or PHI-BLAST, several high scoring hits were obtained. However, when reciprocal queries were subsequently made against the *Aspergillus* genome databases (http://www.broadinstitute.org/annotation/genome/aspergillus_group/MultiHome.html), the LaeA orthologues used were not obviously identified. Since this approach therefore led to potential false positives, we used an iterative phylogenetic strategy to identify the *T. reesei* LaeA orthologue. We used BLASTP to detect LaeA orthologues in fungal species more closely related to the *Aspergilli* (such as *Coccidioides immitis*), then used the identified proteins to look for LaeA orthologues in *Dothidiomycetes*, and used the latter one to the *Sordariomycetes* and finally the *Hypocreaceae*. By this method we arrived at 27 putative LaeA orthologues from *Eurotiomycetes*, *Dothidiomycetes* and *Sordariomycetes*. In order to determine the correct amino acid sequence of the most likely candidate for the *T. reesei* LAE1 protein (Trire2:41617), its cDNA was sequenced, which led to the identification of two additional introns and one exon than predicted from the *T. reesei* genome database and an increased similarity to other fungal LaeA/LAE1 proteins. The GenBank accession number for the cDNA is JN791097.

Phylogenetic analysis of LaeA protein sequences (**Figure 1**) produced a tree whose branching was consistent with established phylogenetic relationships between the various taxa, suggesting orthology of the identified protein sequences. Thus, we consider Trire2:41617 the *T. reesei* LaeA orthologue, which we named LAE1.

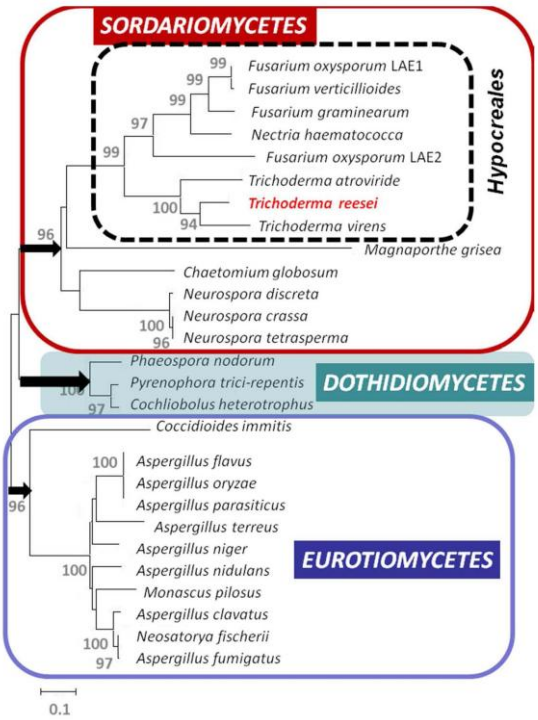

Fig. 1. Phylogenetic analysis of LaeA/LAE1 proteins from *Eurotiomycetes*, *Dothidiomycetes* and *Sordariomycetes.* Accession numbers for protein sequences are listed in Table A3 (Appendix 1). The tree was constructed by Neighbor Joining in MEGA 5.0 (Tamura et al. 2011) with 500 bootstrap replicates (coefficients are indicated below the respective nodes). Gaps in the alignment were not considered.

lae1 is essential for cellulase gene expression in *T. reesei*

To investigate a possible impact of *lae1* on cellulase production in *T. reesei*, *lae1* null mutants (D*lae1*) of the moderate cellulase producing mutant strain *T. reesei* QM 9414 were generated by replacing the *lae1* coding region with the orotidine-5-decarboxylase gene *pyr4* in a KU70-deficient (D*tku70*) strain (Guangtao et al. 2009). Since retransformation of the latter strain was not possible, we investigated the phenotype of several deletion mutants. Their growth on simple carbon sources such as glycerol was similar to that of the parent strain but growth on cellulose was severely impaired (data not shown), implying that *lae1* is required for normal growth on cellulose. To test whether this was caused by a loss of cellulase production, we cultivated the *lae1* deletion strains and the parent strain on lactose, a carbon source that induces cellulase expression, but whose utilization is independent of the action of secreted cellulases (Seiboth et al. 2007). Growth of the parent strains *T. reesei* QM 9414 and Ku70 was indeed similar (**Figure 2A**), but significantly reduced cellulase activities were found in the cultures of the D*lae1* strain (**Figure 2B**). Similar findings were also obtained with

xylan as a carbon source, on which D*lae1* mutants exhibited strongly reduced xylanase activity (data not shown).

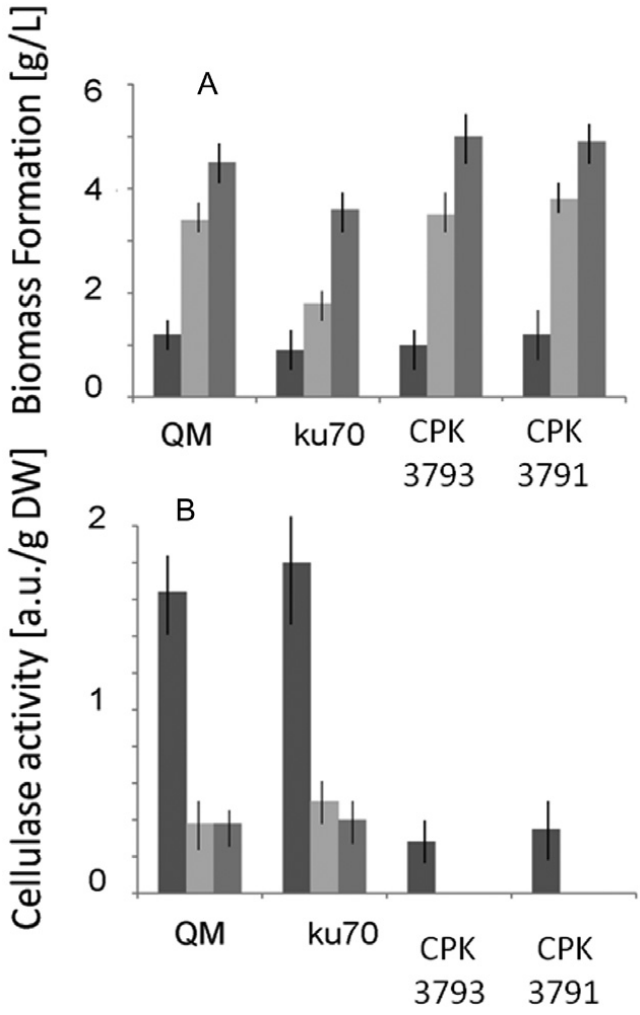

Fig. 2. Effect of loss-of-function of *lae1* on biomass formation and cellulase/hemicellulase enzyme formation by *T. reesei.* Growth (A) and cellulase formation (B) of *T. reesei* QM 9414, the transformation recipient *ku70* and the corresponding D*lae1* strains CPK3793 and CPK3791 on 1% (w/v) lactose. Cellulase expression is given in arbitrary units and related to the respective biomass dry weight of the strain at the respective time point (given in A). The three bars represent (from left to right) values for 48, 72 and 96 h of cultivation. Experiments are means of three biological replicates, and the SD given by vertical bars.

lae1 regulates expression of the CAZyme gene clusters in *T. reesei*

Results described above provided evidence of LAE1 influence on cellulase and hemicellulase expression by *T. reesei*; however, these effects cannot be validated solely from growth data. In order to test whether deletion of *lae1* indeed impairs the expression of biomass-degrading enzymes, we carried out transcriptome analysis. Microarrays representing all 9143 unique predicted ORFs in the *T. reesei* genome were used to determine their relative expression in the wild-type and D*lae1* strains when grown on lactose as a carbon source**.** We found 769 genes with at least a twofold decrease in

their hybridization intensity in the D*lae1* strain compared with the control, QM 9414. Among these, 50 CAZyme-encoding genes were detected, the majority of which comprised glycosyl hydrolases (GH) involved in cellulose and hemicellulose degradation [**Table 1**; see also Table A1 (Appendix 1)].

Table 1. Changes in CAZome gene expression in *T. reesei* after deletion of *lae1*.[a]

	Protein ID	downregulated	p-value
Cellulases			
GH 5 endo-ß-1,4-glucanase CEL5A	120312	17.338	0,000896
GH5 endo-ß-1,4-glucanase CEL5B	82616	5.179	0,00219
GH6 Cellobiohydrolase 2 CEL6A	72567	15.565	0,00106
GH7 endo-ß,4-glucanase CEL7B	122081	3.019	0,00126
GH7 cellobiohydrolase 1 CEL7A	123989	6.841	0,000994
GH12 endo-ß-1,4-glucanase CEL12A	123232	15.789	0,00132
GH45 endo-ß-1,4-glucanase CEL45	49976	14.409	0,000892
GH61 cellulase enhancing protein CEL61A	73643	25.659	0,000851
GH61 cellulase enhancing proteinCEL61B	120961	40.524	0,000836
GH1 ß-glucosidase CEL1B	22197	3.454	0,000918
GH1 ß-glucosidase CEL1A	120749	2.428	0,00107
GH3 ß-glycosidase of uncertain specificity	108671	2.833	0,00142
GH3 ß-glucosidase CEL3D	46816	2.115	0,00525
GH3 ß-glucosidase CEL3C	82227	3.555	0,00153
Nonenzymatic cellulose attacking enzymes			
CIP1	73638	16.794	0,000855
CBM13 protein	111094	8.529	0,00407
Swollenin	123992	3.714	0,000847
swollenin-like, 84 % ID to 123992	111874	6.17	0,00153
Xylanases			
GH10 xylanase XYN3	120229	4.392	0,00202
GH11xylanase XYN1	74223	2.038	0,00213
GH11 xylanase XYN2	123818	23.487	0,000931
GH30 xylanase XYN4	111849	2.121	0,000885
GH3 ß-xylosidase BXL1	121127	17.003	0,000896
GH43 ß-xylosidase/α-arabinofuranosidase	3739	2.752	0,000911
GH74 xyloglucananase CEL74A	49081	3.051	0,0009
Hemicellulose side chain cleaving enzymes			
CE5 acetyl xylan esterase AXE1	73632	6.821	0,000951
GH67 α-glucuronidase AGU1	72526	13.365	0,00101
CIP2 methyl glucuronoyl esterase	123940	3.232	0,00118
GH54, L-α-arabinofuranosidase ABF1	55319	2.201	0,00158
GH95 α-fucosidase	58802	9.946	0,00544
GH95 α-fucosidase	5807	3.606	0,0018
GH92 α-1,2-mannosidase	74198	6.097	0,000712
GH92 α-1,2-mannosidase	60635	2.154	0,00372
GH47 α-1,2-mannosidase	45717	4.496	0,00133
GH2 ß-mannosidase	69245	5.937	0,00181
GH27 α-galactosidase AGL1	72632	5.777	0,00134
GH27 α-galactosidase AGL3	27259	2.065	0,00641
Pectinases			
GH28 polygalacturonase	103049	2.382	0,00831

* Values are given as means of two biological replicates; „downregulation“is given as –fold decrease; CPK3793 was used as *lae1* delta strain.

This included 9 of the 10 cellulases and cellulase-enhancing proteins (CEL61) present in *T. reesei*; only the cellulase-monooxygenase CEL61C (TRire2:27554) was absent from this list. Transcripts for other proteins were also downregulated, such as the swollenin SWO1, a protein carrying an expansin-like

domain that disrupts the crystalline cellulose structure (Saloheimo et al. 2002), CIP1, which contains a signal peptide and a cellulose-binding domain (Foreman et al. 2003, Li et al. 2007), and four xylanases (XYN1 to XYN4). These findings imply that expression of most of the cellulolytic and hemicellulolytic genes is affected by deletion of *lae1*. The majority of the other affected GHs (21 of 28) comprised glycosidases active against various side-chains in hemicelluloses. The reduction of expression of various glycosidase genes was also reflected in the decreased ability to grow on several of their di- or oligosaccharide substrates (**Figure 3**).

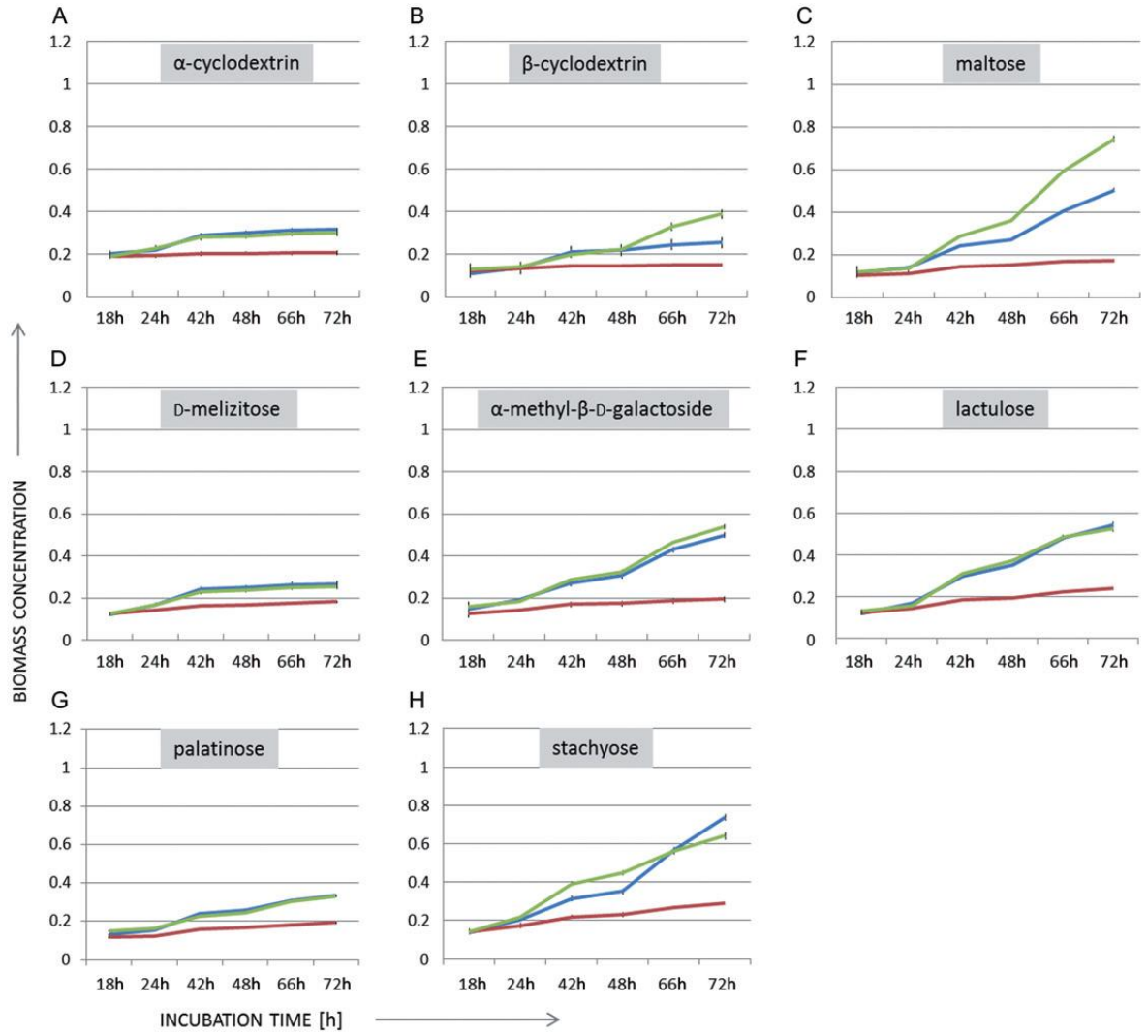

Fig. 3. Growth of *T. reesei* QM 9414 (blue), the D*lae1* strain (CPK3793, red) and the strain expressing *tef1:lae1* (CPK4086, green) on several oligosaccharides. Data were obtained from Phenotype microarrays as described (Druzhinina *et al.*, 2006). Carbon sources used were: (A) a-cyclodextrin, (B) b-cyclodextrin, (C) maltose, (D) D-melizitose [a-D-glucopyranosyl-(1→3)-*O*-b-D-fructofuranosyl-(2→1)-a-D-glucopyranoside], (E) a-methyl-b-D-galactoside, (F) lactulose [4-*O*-b-D-galactopyranosyl-D-fructofuranose], (G) palatinose [6-*O*-a-D-Glucopyranosyl-D-fructose], (H) stachyose [b-D-Fructofuranosyl-*O*-a-D-galactopyranosyl-(1→6)-*O*-a-D-galactopyranosyl-(1→6)-a-D-glucopyranoside]. The vertical axis shows the OD750 that is equivalent to biomass formation (g l-1).

The 25 CAZyme gene clusters in the *T. reesei* genome contain an average fivefold increase in CAZyme gene density compared with the expected density for randomly distributed genes (Martinez et al. 2008). In total, 126 of the approximately 320 CAZyme genes are found in these regions ranging from 14 kb to 275 kb in length. As we identified 769 of the total 9143 genes in the *T. reesei* genome, we would expect one gene with decreased gene expression at every twelfth locus if their distribution would be random. However, if the genes are clustered as calculated above, those with decreased expression should occur in at least every third locus or even be closer to each other. To investigate this, we mapped the 769 identified genes onto the *T. reesei* scaffolds and searched for potential clustering. Indeed, we found 28 regions on 21 scaffolds that exhibited an at least threefold increase of genes with changed expression over the random distribution (**Table 2**). The null hypothesis of random distribution of the clusters was rejected because of the low Pearson coefficient (r = 0.04; t = 0.15). On average, these clusters comprised 7.2 genes and were 6.6-fold enriched over the average. Interestingly, 13 of these 28 clusters were found in areas not previously predicted as CAZyme gene clusters (Martinez et al. 2008).

Table 2. Clusters of expressed genes affected in *T. reesei* D*lae1*.

scaffold	cluster predicted*	found**	genes in cluster	expressed genes	gene density***	CAZys
1	yes	618-690	72	23	3.13	5
	yes	112-135	23	9	2.55	3
2	yes	410-417	7	2	3.5	2
3	no	4-27	23	6	3.83	2
	yes	530-547	17	6	2.83	2
4	no	285-293	8	5	1.6	1
5	no	3-18	15	5	3	1
	yes	209-233	24	8	3	1
6	no	134-138	4	4	1	1
7	no	226-252	26	8	3.25	2
	yes	394-419	25	9	2.77	2
8	yes	167-192	25	7	3.51	3
10	yes	183-201	18	10	1.8	2
13	yes	16-34	18	5	3.6	1
	no	43-61	18	6	3	1
14	no	187-194	7	3	2.33	1
16	no	120 - 134	14	4	3.5	2
19	yes	62-97	35	10	3.5	1
	yes	157-189	32	12	2.67	1
22	no	48-98	50	17	2.94	1
27	yes	40-59	19	5	3.8	1
28	no	68-80	22	7	3.15	1
	no	111-117	6	4	1.5	3
29	yes	91-102	11	7	1.57	2
30	no	4-19	15	4	3.75	1
31	yes	43-53	10	4	2.5	1
33	yes	29-42	13	6	2.16	1
44	no	4-15	11	5	1.2	1

a. Specifies whether the expressed genes were found within the area of clusters proposed by Martinez et al. (2008) or not (in the latter case also shaded). 'Genes in cluster' gives the total number of genes clustered.
b. 'Scaffold gene number' specifies which genes on the scaffold are included in the cluster and refer to the order of genes from the 5´ to the 3´ end of the scaffold.
c. 'Differentially expressed genes/total genes' gives the number of genes from that cluster that are > 2-fold differentially expressed and the total gene number in the cluster.

LAE1 dependence of cellulase gene expression is inducer independent

In order to confirm the results from microarray analysis, we used *cel7A* and *cel6A* as cellulase model genes and tested their expression in the parent strain and in the D*lae1* strain by qRT-PCR. We used cultures grown on lactose or induced with sophorose, a disaccharide conferring high cellulase

induction in resting cells (Sternberg and Mandels, 1979) (**Figure 4**). qRT-PCR data confirmed the results from the microarray experiments, as cellulase gene expression on the two soluble carbon sources was absent in the D*lae1* strains. The results also demonstrate that cellulase regulation by LAE1 is independent of the nature of the inducer.

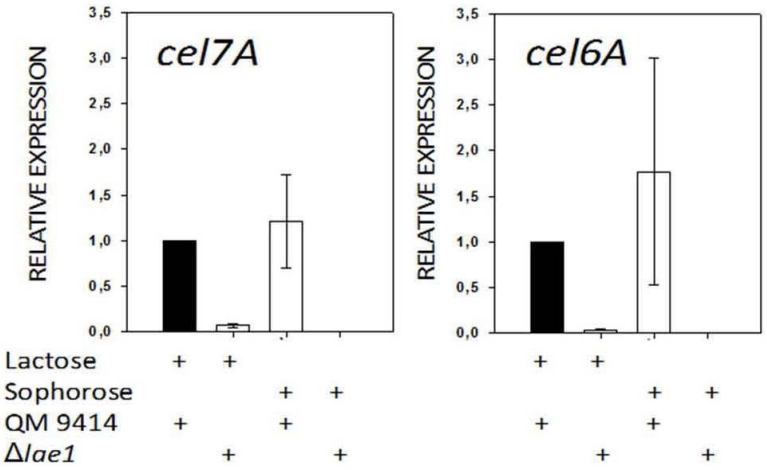

Fig. 4. Expression of the two cellobiohydrolase-encoding genes *cel7a* and *cel6a* in *T. reesei* QM 9414 and the D*lae1* strain CPK3793 during growth on lactose or incubation with sophorose. Cellulase transcript levels in QM 9414 during growth on lactose are given with full bars and set to 1.0. The respective transcript levels in relation to QM 9414 are shown with open bars. Data are means of triplicate determinations from two biological replicates.

Introducing a constitutively expressed *lae1* allele into *T. reesei* enhances cellulase formation

Having identified LAE1 as a regulator of cellulase and hemicellulase biosynthesis in *T. reesei*, we hypothesized that its activity in the parent strain could be limiting for cellulase gene expression. We therefore tested whether increased activity of LAE1 by overexpression of *lae1* would stimulate cellulase formation in the same strain.We fused the *lae1* ORF to the 5´-upstream sequences of the *tef1* (translation elongation factor 1a-encoding) gene, which was expected to result in high constitutive expression of *lae1*. Three *T. reesei* strains that contained either one or two copies of the *tef1:lae1* construct integrated ectopically in the genome were examined for their ability to produce cellulases on lactose. Growth of all transformants was comparable until 72 h of cultivation, but – unlike the wild-type strain – several of them did not start to autolyse thereafter (indicated by the loss in mycelia dry weight; **Figure 5A**). The strains bearing the *tef1:lae1* copies exhibited up to 10-fold increased protein and cellulase formation, which was highest in the strains that did not show autolysis (**Figure 5B** and **C**).

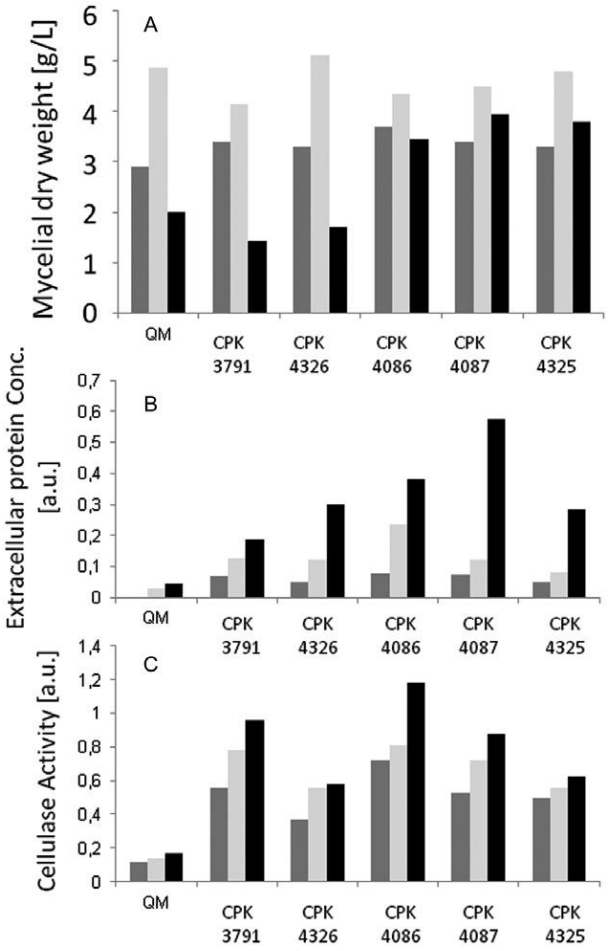

Fig. 5. Biomass formation (A), cellulase production (B) and extracellular protein (C) during growth of *T. reesei* QM 9414 (QM) and several mutant strains bearing additional copies of the *tef1:lae1* gene construct (CPK3791, CPK4326, CPK4086, CPK4087, CPK4325) on lactose. The three bars represent (from left to right) values for 48, 72 and 96 h of cultivation. Each bar is from a single experiment only but representative of at least four biological replica that were consistent with the claims.

We also quantified the expression of *cel7A* and *cel6A* and found them to be 10-to 40-fold increased (**Figure 6**).

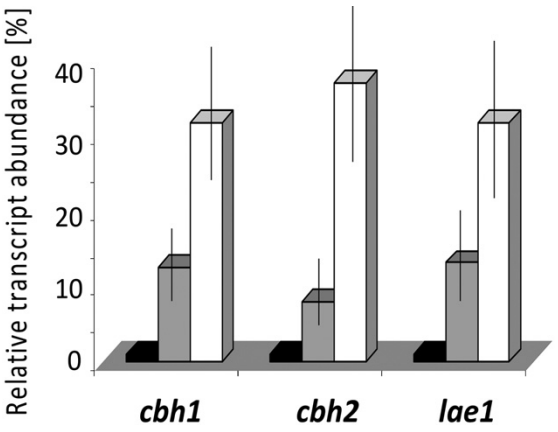

Fig. 6. Relative abundance of transcripts for *cel7a, cel6a* and *lae1* at 26 h of growth on lactose in two *T. reesei tef1:lae1* mutant strains (CPK4086, grey bars; CPK3791, white bars) in relation to the QM 9414 recipient strain (black bars). Transcripts were normalized to the housekeeping gene *tef1*, and the respective ratio in QM 9414 set to 1. Other strains are given in percentage to that of the QM 9414. Copy numbers were determined as described in Materials and Methods: CPK4086 had two additional copies indicating a single ectopically integrated copy; CPK3791, however, had three additional copies, suggesting more than one ectopically integrated copy, but the exact number was not determined. Vertical bars indicate SD.

This increased expression correlated with a five- to eightfold increased expression of *lae1* in these strains. The higher *lae1* expression in CPK3791 may be due to the presence of more than one ectopically integrated *lae1* copy (as in CPK4086) but the exact copy number was not determined. Growth of the *tef1:lae1* mutant strains on cellulose also confirmed increased cellulase formation, although less dramatically than on lactose (**Figure 7**).

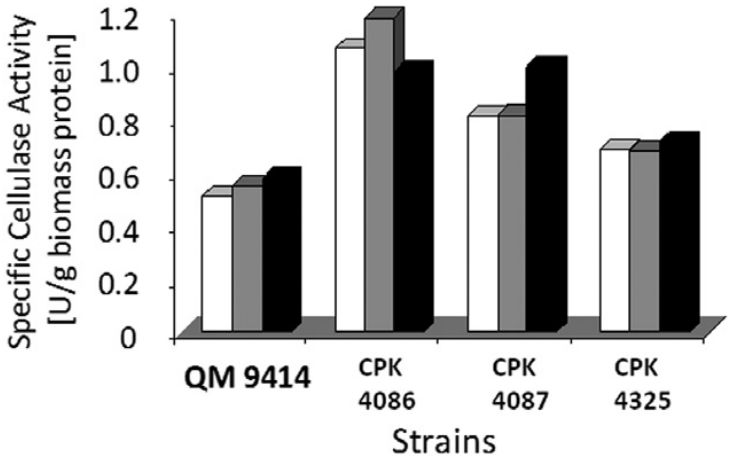

Fig. 7. Cellulase activity during growth of selected *T. reesei tef1:lae1* mutant strains on cellulose. Activities are given as arbitrary units and are related to 1 g of fungal biomass protein. Bars indicate measurements after 7, 9 and 11 days of incubation (from left to right). Data are means from four measurements and two independent biological replicates.

The comparatively smaller effect of *lae1* overexpression on cellulose may be due to the fact that cellulases are required for growth, and their overexpression may lead to an excess of cellobiose and glucose formed that in turn induces carbon catabolite repression of cellulase expression. Microarray expression analysis revealed 68 CAZyme genes to be significantly upregulated (> 2-fold) in the *tef1:lae1* strain CPK4086 [Table A1 (Appendix 1)]. A comparison with the CAZyme genes that were downregulated in the D*lae1* strain showed that 27 genes were consistently affected in both strains, i.e. exhibited strongly reduced expression in the D*lae1* strain but enhanced expression in the *tef1:lae1* strain (**Figure 8**).

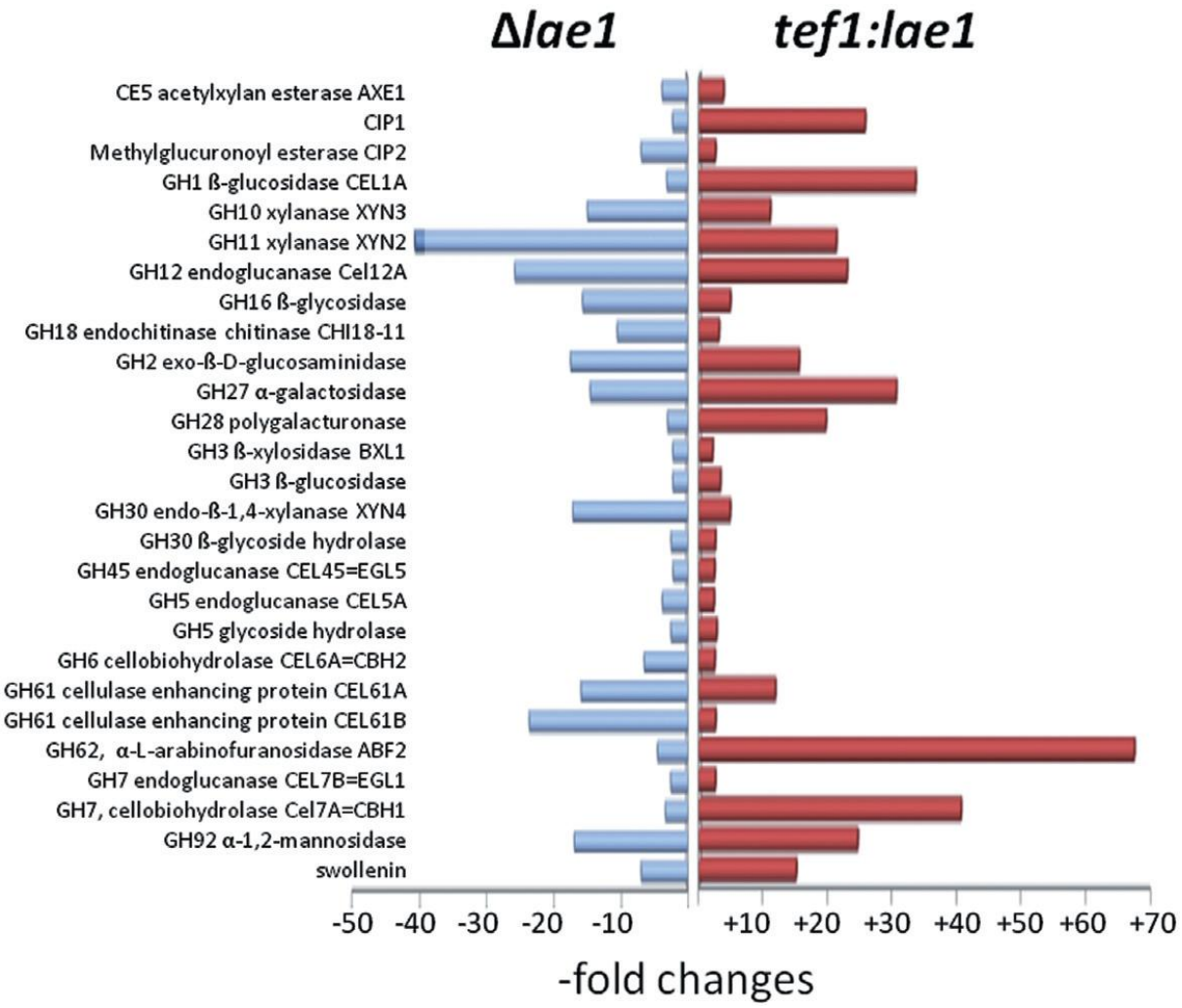

Fig. 8. Relative changes in expression of CAZome genes in the D*lae1* (CPK3793) and the *tef1:lae1* (CPK4086) strains, given as the fold changes of transcript hybridization in relation to the parent strain. Only values with $P < 0.05$ are shown.

These genes included eight of the 10 cellulases and cellulase-enhancing proteins (see above), the xylanases XYN1, XYN2, XYN3 and XYN4, and the auxiliary factors CIP1 and swollenin. The remaining genes comprised various b-glycosidases active on hemicelluloses side-chains. Interestingly, CAZyme genes that responded only to *lae1* overexpression included eight chitinases, six b-glucanases and two CBM13 carbohydrate binding modules, suggesting that *lae1* regulation of CAZyme gene expression may play roles beyond cellulose degradation.

Regulation of expression of cellulases by *lae1* is dependent on the function of XYR1

The above data suggest that regulation of cellulase and hemicellulase gene expression cannot bypass the necessity of an inducer. In order to investigate this at a molecular level, we replaced the *xyr1* gene, encoding the major cellulase and hemicellulase regulator XYR1 (Stricker et al. 2006), by a constitutively expressed *pki1:xyr1* allele in the D*lae1* strain. Likewise, we deleted *xyr1* in the *lae*1OE strain. The data, shown in **Figure 9**, demonstrate that neither the overexpression of *lae1* in a D*xyr1* background, nor the overexpression of *xyr1* in a delta-*lae1* background resulted in cellulase formation. Increased transcription of *xyr1* and *lae1* from the constitutive promoters was proven by qPCR (data not shown). These data are consistent with the inducer dependence of *lae1* overexpression, and demonstrate that *lae1* requires the function of *xyr1*, but also that *xyr1* depends on the function of *lae1*.

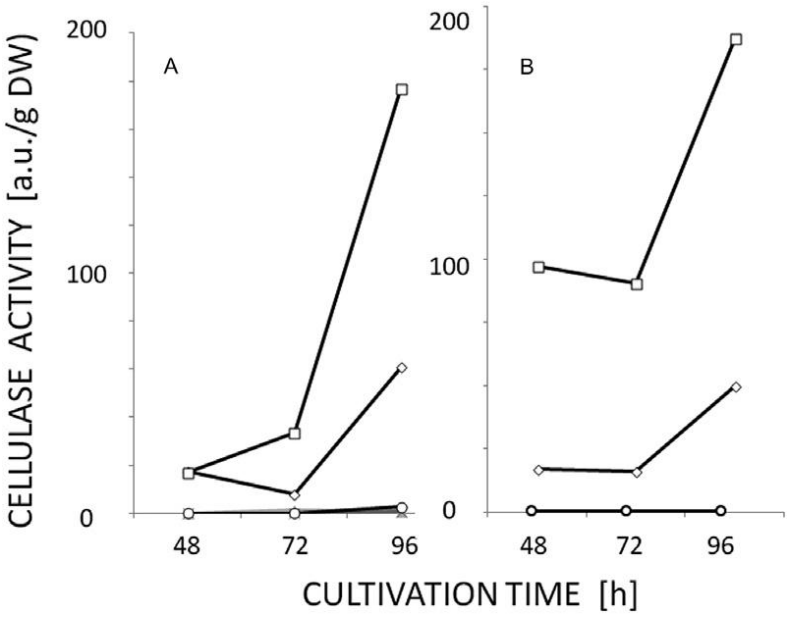

Fig. 9. Cellulase formation in delta-*lae1* (CPK3793) and delta-*xyr1* strains of *T. reesei* is unaffected by the constitutive overexpression of *xyr1* or *lae1*.
A. Overexpression of *xyr1* in a delta-*lae1* background: three different transformants yielded identical results and thus shown only for one of them (circles). The wild-type QM 9414, and QM 9414 containing a single *pki1:xyr1* copy are given as comparison (diamonds and squares respectively). B. Overexpression of *lae1* in a delta-*xyr1* background: three different transformants yielded again identical results and thus shown only for one of them (circles). The wild-type QM 9414, and strain CPK4086 overexpressing *tef1:lae1* are given as comparison (diamonds and squares respectively). Data from a single experiment only are shown, but are consistent with the results from at least two separate biological replicas.

Expression of CAZyme genes in D*lae1* and *tef1:lae1* is not correlated with methylation at histone 3 lysine 4 or lysine 9

Expression of secondary metabolism gene clusters in *Aspergillus* is affected by LaeA (Bok and Keller, 2004, Bayram et al. 2008), presumably by changing specific histone modifications (see review by Strauss and Reyes-Dominguez, 2011). Direct biochemical evidence for this hypothesis is still lacking. To test this hypothesis for regulation of the CAZyme genes in *T. reesei*, we performed chromatin immunoprecipitation followed by highthroughput sequencing ("ChIP-seq") on wild type, D*lae1* and *tef1:lae1* strains that had been grown in lactose containing medium for 26 h. Histone H3 lysine 4 (H3K4) dimethylation is indicative of the potential for transcription and H3K4 trimethylation (H3K4me3) suggests active transcription of genes associated with nucleosomes that carry this modification (Hublitz et al. 2009). H3K9me3 is associated with gene silencing, typically in regions of facultative or constitutive heterochromatin (Lewis et al. 2009). ChIP with H3K4me2, H3K4me3 and H3K9me3 antibodies resulted in the expected patterns in most transcribed genes for all three strains examined in this study, i.e. H3K4me2 and H3K4me3 enrichment and absence of H3K9me3 (a detailed, genome-wide analysis of the results is in preparation). However, only one CAZyme gene (*cel5B*) showed enrichment of H3K4 methylation in wild type and *tef1:lae1*, with concomitant reduction in D*lae1*, and one gene (*cel1A*) showed some enrichment of H3K4me2 in all strains without enrichment of H3K4me3 (**Figure 10A**). There was no clear change in histone modifications within any of the CAZyme genes that we showed by microarray analysis to have expression levels that are strongly affected by either the absence or overexpression of LAE1. As expected, several CAZyme genes showed minor enrichment of H3K4 methylation in the *tef1:lae1* overexpression strain (*xyn2*, *cip1*, *cel6A*, *cel7B*, *cel61B*; data not shown). However, the most commonly observed pattern for these genes (*cel12A*, *cel61A*, *cel45A*, *cel3D*, *cel3C*, *cel5A*, *cel7A*, 108671/GH3 β-glycosidase, 111874/swollenin-like, *cbm13*, 123992/swollenin, *xyn1*, *xyn3*, *cip2*; see *cel1B*, **Figure 10B**) was no enrichment with any of the histone marks we tested. Also, no enrichment was seen at the *xyr1* locus

(data not shown). ChIP-seq results were validated by region-specific PCR of most regions and results were similar, i.e. no enrichment differences for any of the histone marks in the three different strains in any of the regions tested (data not shown). The ChIP-seq was successful; as most genes showed the expected patterns for H3K4 and H3K9 methylation (e.g. see the genes for histone H3 and H4-1, and ornithine decarboxylase; **Figure 10C** and **D**). Also, the *lae1* gene served as a convenient control as in D*lae1* there was no enrichment of any kind observed (the flat line indicates absence of the segment from the genome; **Figure 10E**), while in *tef1:lae1* both H3K4me2 and H3K4me3 were enriched at *lae1*, which is consistent with overexpression of the gene. Lastly, one gene for a putative carbohydratebinding protein (protein ID 102735) was more highly enriched for H3K4me3 in D*lae1* but not in any other condition. Taken together, this analysis suggests that LAE1 does not directly affect the balance of H3K4 and H3K9 methylation at the CAZyme genes in *T. reesei*.

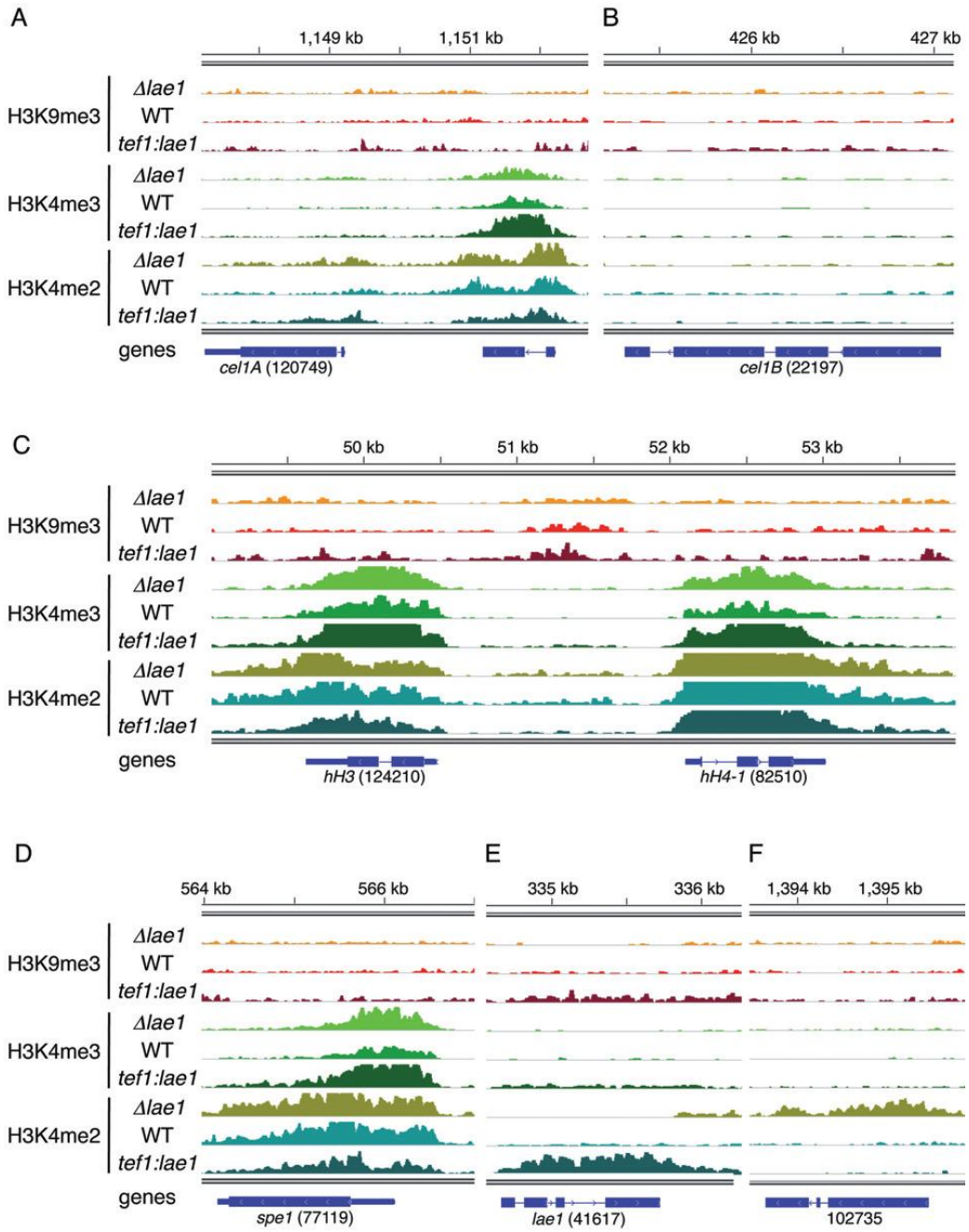

Fig. 10. Selected results of ChIP-seq with antibodies against H3K9me3, H3K4me3 and H3K4me2 in D*lae1* (CPK3793), wild type (QM 9414) and the LAE1-overexpressing *tef1:lae1* strain CPK4086. Genes of interest are shown below regions of enrichment (*y*-axis scale, 0–30 for all tracks, all data were normalized for relative abundance).

A. H3K4me3 is mildly enriched in the 5´ region of *cel1a* but not nearly as strongly as in the neighbouring gene, which is not affected by changes in LAE1 expression.

B. The most common pattern of CAZyme gene histone modifications tested here was no significant enrichment under any condition, represented here by *cel1b*.

C. In contrast, highly expressed genes, like *hH3* and *hH4-1* show both H3K4me2 and -me3 but no H3K9me3 enrichment in a *lae-1*-independent manner.

D. The same is true for a typical metabolic gene, *spe1*, the gene for ornithine decarboxylase.

E. The *lae1* gene serves as control, as no signal is detected in D*lae1*, but H3K4me2 is enriched in the *tef1:lae1* strain.

F. A predicted gene encoding a protein with a carbohydrate-binding motif (protein ID 102735) shows enrichment of H3K4me2 only in the D*lae1* strain.

LAE1 is essential for *T. reesei* asexual sporulation

Manipulation of *lae1* gene expression in *T. reesei* caused striking phenotypic changes: delta-*lae1* strains can readily be observed on plates by the lack of the characteristic yellow pigment that is produced by *T. reesei*, and by the significantly reduced sporulation (**Figure 11**). Overexpression of *lae1* (OE*lae1*) recovered the pigmentation, and led to even increased sporulation (**Figure 11A**). A quantitative analysis showed that sporulation was reduced to 5% of the control in delta-*lae1*, and doubled in the OE*lae1* strain compared with the control (**Figure 11B**). Interestingly, sporulation in the wild type and the delta-*lae1* strain was not effected by light or darkness, but the increased sporulation in the OE*lae1* strain was only apparent in light.

We consequently wondered whether this difference would also be reflected in a similarity between the conidiating and *lae1*-effected transcriptome. Metz et al. (2011) have recently reported that 900 genes are significantly expressed in *T. reesei* during conidiation. When they were compared with the transcriptome of the delta-*lae1* strain, 254 genes (i.e. 47.9% of all genes upregulated during conidiation and 33.2% of the genes found to be downregulated in delta-*lae1*) correlated between the two conditions (i.e. upregulated during sporulation and downregulated in the delta-*lae1* strain; and vice versa) [Table A2 (Appendix 1)].

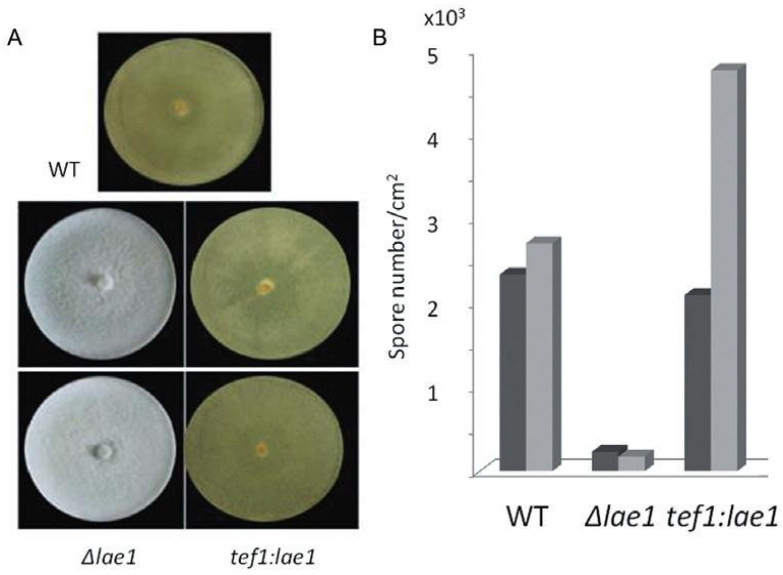

Fig. 11. LAE1 affects sporulation in *T. reesei*. (A) Phenotype of *T. reesei* QM 9414 (WT), D*lae1* (CPK3793 on top, and CPK4086 below) and *lae1*OE strains (CPK4086) on top, CPK3791 below); (B) effect of light on sporulation in *T. reesei* WT, D*lae1* (CPK3793) and *lae1*OE (CPK4086) strains. Only one strain is shown, but consistent data have been obtained with at least two more strains of both *lae1* genotypes.

Discussion

Data presented here show that the putative protein methyltransferase LAE1 influences cellulase gene transcription in *T. reesei*, and may thus represent a novel approach for cellulase overproduction and strain improvement by recombinant techniques in this fungus. Although the use of LaeA for increasing secondary metabolite production by fungi has been proposed (Keller and Bok, 2006, Keller et al. 2007), we are not aware of any successful demonstration of this principle in an industrially relevant fungal species. Since we have used one of the "early" strains from the *T. reesei* mutant pedigree, *T. reesei* QM 9414 (Le Crom et al. 2009), we cannot be certain that high-cellulase producing strains exhibit the same degree of increase in cellulase formation as observed here with strains that constitutively expressed *lae1*. However, we note that genome-wide analysis of the currently highest cellulase producing strain in the public domain – *T. reesei* RUT C30 – did not reveal mutations in genes related to chromatin-level gene regulation, and its *lae1* locus was intact (Le Crom et al. 2009). Our data suggest an attractive new approach for increasing total cellulase activity in *T. reesei* in a single step. The only other means to globally increase production of all cellulases – by increasing the expression or activity of the cellulase- and hemicellulasespecific transcriptional regulator XYR1 (Stricker et al. 2006) – has so far not yielded significant improvements (Mach-Aigner et al. 2008).

Interestingly, *xyr1* itself is located in one of the CAZyme clusters, and downregulated in the D*lae1* strain during growth on lactose [Table A1 (Appendix 1)]. Data from this article further showed that overexpression of either *xyr1* or *lae1* under constitutive promoters cannot rescue the impairment of cellulase gene expression by a deletion in the other gene respectively. This suggests that LAE1 is involved in control of transcription of the cellulase genes and that of their common regulator. The signalling pathway by which the presence of any inducer of cellulase activity is communicated to the *T. reesei* transcriptional machinery is not known yet. Its identification will shed light on how LAE1 functions in the activation of transcription of cellulase-encoding genes in this fungus.

While the effect of LAE1 on *T. reesei* CAZyme gene expression has clearly been demonstrated in this article, one hypothesis was falsified, i.e. that genomic clusters of cellulase genes (Martinez et al. 2008) would serve as targets for LAE1-mediated counteraction of H3K9 methylation, and thus binding of HP1 and generation of heterochromatin. In fact, the LAE1-independent absence of H3K9me3 from the CAZyme loci, and the very slight enrichment of H3K4 methylation in LAE1 overproducing *T. reesei* mutants suggests that the cellulase genes are only little affected by repressive and non-repressive chromatin, and thus LAE1 activates CAZyme gene transcription by a mechanism independent of histone H3K4 and H3K9 methylation. Unfortunately, the target protein of methylation by LAE1 has not yet been identified in any organism (Bayram and Braus, 2012). We must also note that – although ChIP analysis of heterochromatic marks in *A. nidulans laeA*D strains had revealed a dramatic increase in H3K9me3 and HepA binding at secondary metabolism clusters (Reyes-Dominguez et al. 2010) – a direct effect of LaeA on histone modification still awaits biochemical evidence.

While the precise mechanism of action of LAE1 remains to be identified, we speculate that it could be related to the linkage between asexual sporulation and CAZyme gene transcription in *T. reesei* (Metz et al. 2011): as we have shown in this article, one of the most striking phenotypes of *lae1*-mutants in *T. reesei* is the almost complete absence of sporulation in the D*lae1* and hypersporulation

in *tef1:lae1* strains, a phenomenon already observed in some other fungi (Bok et al. 2005, Kosalková et al. 2009). The fact that approximately half of the genes upregulated during asexual sporulation are identical to a third of the genes downregulated in delta*lae1* renders asexual development a major target in *T. reesei*. Asexual sporulation triggers massive CAZyme gene expression in *T. reesei* in an inducer-independent but XYR1-dependent way (Metz et al. 2011), and sporulation is commonly observed at later stages of cellulase formation on lactose which was absent from the D*lae1* strain and increased in the *tef1:lae1* mutant in this study (data not shown). Absence of conidial cellulases renders them unable to germinate on cellulose as a carbon source (Metz et al. 2011). We consider it therefore possible that the regulation of sporulation is the prime target of LAE1, and that the effect on cellulase gene expression occurs by a signal for CAZy gene expression created during sporulation. We should note that sporulation is also commonly observed during submerged growth of *Trichoderma* spp. on carbon sources inducing cellulase formation such as cellulose or lactose (Lewis and Papavizas, 1983).

Independent of the underlying mechanism, our results demonstrate the obvious advantage of genomic clustering of the *T. reesei* cellulases, although the evolutionary mechanisms that have led to this situation still remain unclear. In the case of secondary metabolite biosynthetic genes, clustering has been suggested to reflect their evolutionary history (Zhang et al. 2004, Keller et al. 2005). One model, the “selfish cluster” hypothesis, requires that selection occurs by promoting the maintenance of the cluster as a unit, e.g. by horizontal transfer events (Walton, 2000). As far as the major cellulases and hemicellulases of *T. reesei* are concerned; however, there is no evidence or indication for horizontal gene transfer. Alternatively, it has been suggested that clusters are maintained by the operation of co-regulatory mechanisms. This is essentially what is observed with cellulases and hemicellulases, which are co-induced by similar signals (Foreman et al. 2003) and controlled by a single major transcriptional regulator XYR1 (Stricker et al. 2006). In summary this article describes the successful attempt to increase production of all *T. reesei* cellulases by modulation of a single gene.

While reaching a cost-efficient application of second-generation biofuel production still depends also on an improvement of several of the steps involved, e.g. biomass pre-treatment, enzyme composition and pentose fermentation (Margeot et al. 2009, Xu et al. 2009), our data suggest a means to solve one of the essential steps, i.e. the need for increasing enzyme production. Learning how LAE1 becomes active and identifying its actual target protein in *T. reesei* will contribute additional or alternative tools for improvement of cellulase production at the chromatin level.

Materials and Methods

Strains used in this work and their cultivation

Trichoderma reesei QM 9414 (ATCC 26921), an early cellulase producing mutant and *T. reesei* KU70 (Guangtao et al. 2009), a derivative of the QM 9414 uridine auxotrophic *pyr4*-negative strain TU-6 (ATCC MYA-256) (Gruber et al. 1990) which bears a deletion in the *tku70* gene and is thus deficient in non-homologous end joining, were used throughout this work. *Escherichia coli* JM109 (Promega, Madison, Wisconsin) was used for plasmid construction and amplification.

For cellulase production, *T. reesei* was grown in Mandels-Andreotti medium (Akel et al. 2009) using Avicel cellulose, lactose, oat spelts xylan or glycerol as a carbon source (1%, w/v) as stated at the respective results. Induction of cellulases by sophorose (0.5 mM) in pre-grown, washed mycelia was performed as described (Sternberg and Mandels, 1979).

Phylogenetic identification of *T. reesei* LAE1

For the identification of the *T. reesei* LaeA orthologue the LaeA proteins from *A. nidulans* and *A. fumigatus* were used to retrieve the respective orthologues from all other *Aspergilli* by BLASTP. These proteins were then collectively used to retrieve the *C. immitis* LaeA. The latter was used to identify the corresponding orthologues from the three *Dothidiomycete* taxa *Phaeospora nodorum, Pyrenophora tritici repentis* and *Cochliobolus heterostrophus*. Using the latter, we identified the

proteins from the three *Neurospora* spp. followed by the identification of the LaeA orthologues in *Chaetomium globosum* and *Fusarium* spp. before we finally identified the LaeA orthologues in the three *Trichoderma* spp. The correctness of the best hits was always cross-checked by reversed BLASTP and these best hits always exhibited a reliable aa-identity of > 70%. For gene/protein sequences used in this approach, see Table A3 (Appendix 1).

Nucleic acid isolation and hybridization

Fungal mycelia were harvested by filtration, washed with distilled cold water, frozen and ground under liquid nitrogen. For extraction of genomic DNA, plasmid DNA and RNA, purification kits (Wizard Genomic DNA Purification Kit, PureYield Plasmid Midiprep System and SV Total RNA Isolation System, respectively, all from Promega) were used according to the manufacturer's protocol. cDNA synthesis from the predicted *lae1* mRNA was done with a RevertAid^{TM} H Minus First strand cDNA Synthesis Kit (Fermentas, MA), using the primers Lae1ATG and LAE1stop [Table A4 (Appendix 1)]. Standard methods were used for electrophoresis, blotting and hybridization of nucleic acids.

Construction of *T. reesei* strains with altered *lae1* alleles

To study the function of LAE1, we constructed *T. reesei* strains in which *lae1* was deleted and strains which expressed *lae1* under the strong constitutive expression signals of the *tef1* (translation elongation factor 1-alpha encoding) promoter region (Akel et al. 2009).

To delete the *lae1* gene of *T. reesei,* the 1.2 kb *lae1* coding region was replaced by the *T. reesei pyr4* (orotidine 5´-phosphate decarboxylase-encoding) gene. This was performed by amplifying around 1 kb of the up- and downstream non-coding region of *lae1* from genomic DNA of *T. reesei* QM9414 using the primer pairs given in Table A4 (Appendix 1). The two resulting PCR fragments were digested with *Hind*III/*Xho*I (upstream region) and *Apa*I/*Xho*I (downstream region) and ligated into an

ApaI/*HindIII* restricted vector pBluescript SK(+) (Stratagene, La Jolla, California), followed by the insertion of the 2.7 kb SalI fragment of *T. reesei pyr4* in the *Xho*I site resulting in pRKA_D41617pyr4.

For expression of *lae1* under a strong constitutive promoter in *T. reesei* QM 9414 and in the *T. reesei* D*xyr1* strain (Stricker et al. 2006), we amplified a 1820 bp *lae1* PCR fragment including the coding and terminator region with the oligonucleotides TrLae1ATGCla and TrLae1TermHind [Table A4 (Appendix 1)] and inserted the fragment downstream of the *tef1* promoter region (GenBank Accession No. Z23012.1) into the *Cla*I/*Hind*III sites of pLH1hphtef1 resulting in vector pRKA_OE41617hph, which contains the *E. coli* hygromycin B phosphotransferase (*hph*) under *T. reesei* expression signals as selection marker (Akel et al. 2009).

To construct a strain constitutively expressing *xyr1* in a delta-*lae1* background, the plasmid pRLMex30 (Mach et al. 1994) was digested with *Xba*I and *Hind*III, removing the *hph* coding region and *cbh2* terminator region (2066 bp) and leaving the *pki1* promoter. Next a 2.4 kb fragment containing about 800 bp of the *trpC* promoter, the *nptII* coding region and about 700 bp of the *trpC* terminator was amplified from pII99 (Namiki et al. 2001) using the primers GenFW (CCTCTTAACCTCTAGACGGCTTTGATTTCCTTCAGG) and GenRV (TGATTACGCCAAGCTTGGATTACCTCTAAACAAGTGTACCTGTG). The two fragments were joined by In-Fusion recombination (Clontech) resulting in the plasmid pPki-Gen, which was verified by digestion with *Sac*II and *Xba*I+*Hind*III. Then the *xyr1* coding and terminator region was amplified using the primers XyrFW (CCTCTTAACCTCTAGAATGTTGTCCAATCCTCTCCGTCG) and XyrRV (ATCAAAGCCGTCTA GATCTACAGCCATGCTCATCGTGC). The resulting 3.5 kb fragment was inserted into the *Xba*I site of pPki-Gen by In-Fusion recombination (Clontech) yielding plasmid pPki-Xyr which was verified by digestion with *Xba*I and sequencing. This plasmid was then used to transform the delta-*lae1* strain of *T. reesei*.

Fungal transformation

All vectors constructed were verified by sequencing. The strains were purified twice for mitotic stability, and integration of the expression cassettes was verified by PCR analysis. Protoplast preparation and DNA mediated transformation was described (Guangtao et al. 2009).

Gene copy numbers of the integrated constructs were determined by Southern analysis, using chromosomal DNA cleaved with BamHI. This enzyme cleaves within *lae1*, and at the native locus gives rise to two fragments (2.3 and 4 kb respectively; using the *lae1* cDNA as a probe). No additional BamHI sites occur in the *tef1:lae1* gene construct. Thus in case of an intact wild-type locus, integrated *tef1:lae1* copies are visible by additional pairs of *lae1* restriction fragments.

Biochemical assays

Cellulase enzyme activities were determined using carboxymethylcellulose (1%, w/v) as described (Vaheri et al. 1979). Protein concentrations in the culture supernatant were determined by the method of Bradford (1976).

Transcriptome analysis of *lae1* loss of function and *lae1* overexpression

Mycelia were ground in liquid nitrogen using a mortar and pestle. Total RNAs were extracted using TRIzol® reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions, and then purified using the RNeasy MinElute Cleanup Kit (Qiagen, Hilden, Germany). The RNA quality and quantity were determined using a Nanodrop spectrophotometer. High-quality purified RNAs were submitted to Roche-NimbleGen (40 mg per three-microarray set) where cDNAs were synthesized, amplified and labeled and then used for subsequent hybridization.

A high density oligonucleotide (HDO) microarray (Roche-NimbleGen, Madison, WI, USA) was constructed, using 60-mer probes representing the 9.129 genes of *T. reesei*. Microarray scanning, data acquisition and identification of probe sets showing a significant difference (P = 0.05) in

expression level between the different conditions were performed by Roche-NimbleGen (http://www.nimblegen.com). Values were normalized by quantile normalization (Bolstad et al. 2003) and the RMA algorithm (Irizarry et al. 2003); this was done by Nimblegen. After elimination of transcripts that exhibited an SD > 20% within replicates, the FDR (Benjamini Hochberg) method (Benjamini and Hochberg, 1995) was used to assess the significance of values. Transcripts showing significantly downregulated expression in the D*lae1* strain (at least twofold changes) were annotated manually. The data set was also manually screened for the downregulation of genes encoding carbohydrate active enzymes to at least twofold changes. Gene accession numbers were annotated according to version 2 of the *T. reesei* genome assembly (http://genome.jgi-psf.org/Trire2/Trire2.home.html), and ambiguous cases annotated manually. The microarray data and the related protocols are available at the GEO website (http://www.ncbi.nlm.nih.gov/geo/) under Accession No. GSE22687 (platform GPL10642).

Analysis of genomic clustering of transcripts

Trichoderma reesei genes have not yet been mapped to chromosomes, but their appearance on genomic scaffolds is known. In order to identify whether the significantly regulated transcripts would be clustered to particular areas on these scaffolds, we aligned them onto an ordered list of genes on the individual scaffolds. Distances (= numbers of genes) between positive hits were recorded. Clustering of transcripts was considered to appear if the distance between them was at least threefold smaller than the average distribution of the 769 significantly regulated transcripts among all genes (9143), i.e. a third of 11.9 = 3.9.

Real-time PCR

DNase treated (DNase I, RNase free; Fermentas) RNA (5 mg) as reverse transcribed with the RevertAid™ First Strand cDNA Kit (Fermentas) according to the manufacturer's protocol with a

combination of oligo-dT and random hexamer primers [Table A5 (Appendix 1)]. All real-time RT-PCR experiments were performed on a Bio-Rad (Hercules, CA) iCycler IQ. For the reaction the IQ SYBR Green Supermix (Bio-Rad, Hercules, CA) was prepared for 25 ml assays with standard $MgCl_2$ concentration (3 mM) and a final primer concentration of 100 nM each. All assays were carried out in 96-well plates. Determination of the PCR efficiency was performed using triplicate reactions from a dilution series of cDNA (1; 0.1; 0.01; 0.001). Amplification efficiency was then calculated from the given slopes in the IQ5 Optical system Software v2.0. Expression ratios were calculated using REST© Software (Pfaffl et al. 2002). All samples were analysed in at least two independent experiments with three replicates in each run.

Chromatin immunoprecipitation (ChIP) and ChIP sequencing

To carry out ChIP sequencing with *T. reesei* we adapted a protocol developed for *Neurospora crassa* (Tamaru et al. 2003, Smith et al. 2011). QM 9414, D*lae1* and *tef1::lae1* strains were grown for 5 days in the dark on 2% PDA medium and spores then harvested. Flasks with 50 ml of lactose medium were inoculated with either 1 X 10^5 or 1 X 10^6 spores ml^{-1} and grown in the dark for 26 h. All further steps were as described previously (Tamaru et al. 2003). DNA obtained by ChIP was suspended in 30 ml and used either for region-specific ChIP with primers for CAZyme genes (sequences available upon request) or for the construction of ChIP-seq libraries (Pomraning et al. 2009). We obtained 1.4–4.8 million mapped reads (between 76% and 98% of the total reads) for the nine libraries we sequenced (three strains X three antibodies). The antibodies used were from Active Motif (H3K4me3, 39159; H3K9me3, 39161) and Upstate/Millipore (H3K4me2, 07-030).We used one additional H3K9me3 antibody from abcam (ab8898), which resulted in less enrichment than with the Active Motif antibody. Genomewide analysis of the ChIP-seq results is in preparation.

Statistical analysis

Basic statistical methods such as multiple regression analysis and analysis of variance (ANOVA) as well as multivariate exploratory techniques (cluster and factor analyses) were performed using STATISTICA 6.1 (StatSoft, Tulsa, OK, USA) data analysis software system.

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Chapter 4

Functional Analyses of *Trichoderma reesei* LAE1 Reveal Conserved and Contrasting Roles of this Regulator

The results presented in this chapter have been published
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Abstract

The putative methyltransferase LaeA is a global regulator that affects the expression of multiple secondary metabolite gene clusters in several fungi, and it can modify heterochromatin structure in *Aspergillus nidulans*. We have recently shown that the LaeA ortholog of *Trichoderma reesei* (LAE1), a fungus that is an industrial producer of cellulase and hemicellulase enzymes, regulates the expression of cellulases and polysaccharide hydrolases. To learn more about the function of LAE1 in *T. reesei*, we assessed the effect of deletion and overexpression of *lae1* on genome-wide gene expression. We found that in addition to positively regulating 7 of 17 polyketide or nonribosomal peptide synthases, genes encoding ankyrinproteins, iron uptake, heterokaryon incompatibility proteins, PTH11-receptors, and oxidases/monoxygenases are major gene categories also regulated by LAE1. chromatin immunoprecipitation sequencing with antibodies against histone modifications known to be associated with transcriptionally active (H3K4me2 and -me3) or silent (H3K9me3) chromatin detected 4089 genes bearing one or more of these methylation marks, of which 75 exhibited a correlation between either H3K4me2 or H3K4me3 and regulation by LAE1. Transformation of a *laeA*-null mutant of *A. nidulans* with the *T. reesei lae1* gene did not rescue sterigmatocystin formation and further impaired sexual development. LAE1 did not interact with *A. nidulans* VeA in yeast two-hybrid assays, whereas it interacted with the *T. reesei* VeA ortholog, VEL1. LAE1 was shown to be required for the expression of *vel1*, whereas the orthologs of *velB* and *vosA* are unaffected by *lae1* deletion. Our data show that the biological roles of *A. nidulans* LaeA and T. *reesei* LAE1 are much less conserved than hitherto thought. In *T. reesei*, LAE1 appears predominantly to regulate genes increasing relative fitness in its environment.

Introduction

The *Aspergillus nidulans* LaeA protein, a putative S-adenosylmethionine-dependent (SAM) methyltransferase, was originally described as a global regulator of secondary metabolism (Bok and

Keller 2004). It was later shown to be required for the biosynthesis of secondary metabolites in *Aspergillus* (Bok et al. 2006a, Bouhired et al. 2007, Perrin et al. 2007, Kale et al. 2008, Georgianna et al. 2010, Oda et al. 2011), in the industrial fungus *Penicillium chrysogenum* (e.g., penicillin) and the phytopathogenic *Fusarium fujikuroi* (e.g., gibberellins) and *Cochliobolus heterostrophus*, respectively (Hoff et al. 2010, Wiemann et al. 2010, Butchko et al. 2012, Wu et al. 2012). LaeA acts in a complex with VeA, i.e., Velvet, and VelB i.e., Velvet-like B (Bayram et al. 2008a). Because LaeA was shown to control a region with discrete borders, encompassing 70 kb of the sterigmatocystin cluster in *A. nidulans*, an epigenetic control function of LaeA was postulated (Bok et al. 2006b), further defined as somehow counteracting H3K9 methylation in the sterigmatocystin gene cluster (Reyes-Dominguez et al. 2010). More recently evidence emerged that LaeA also controls developmental events, such as conidiation in numerous fungi (Bok et al. 2005, Sugui et al. 2007, Hoff et al. 2010, Wiemann et al. 2010, Chang et al. 2012, Jiang et al. 2012, Wu et al. 2012), including a light-dependent effect on asexual development in *A. nidulans* (Sarikaya Bayram et al. 2010). Moreover, the absence of LaeA results in the formation of significantly smaller fruiting bodies in *Aspergillus flavus* (Amaike and Keller 2009) and *A. nidulans*, the latter associated with a decrease in the formation of specific globose (or "Hülle") cells, which nurse the young fruiting body during development (Carvalho et al. 2002). Thus, current knowledge suggests that LaeA has a dynamic role in both fungal morphological and chemical development. It is, however, still unknown whether these roles for Lae proteins are uniformly distributed throughout the Pezizomycota. We have recently shown that LAE1, the *Trichoderma reesei* ortholog of *Aspergillus* LaeA, controls the expression of polysaccharide hydrolytic enzymes (Seiboth et al. 2012). *T. reesei* is a saprophyte specialized on pre-degraded wood (Druzhinina et al. 2011), thus suggesting a potential nutritional and eco-physiological role of LAE1 for this species. Interestingly, the control of expression of the polysaccharide hydrolase genes by LAE1 does not appear to involve changes in H3K9 methylation (Seiboth et al. 2012) as it does for some secondary metabolite gene clusters in *A. nidulans* (Reyes-Dominguez et al. 2010). Here we have extended our earlier findings to

the genome-wide scale, with the goal to identify targets of LAE1 function in *T. reesei*, and we compare known functions of *A. nidulans* LaeA and *T. reesei* LAE1.

Results

Genome-wide analysis of LAE1 function

to identify genes that are influenced by LAE1 function in *T. reesei*, we determined the transcriptional profiles of the wild-type, Δ*lae1*, and *lae1*OE strains by comparisons of relative transcript levels between Δ*lae1* and *lae1*OE, respectively, vs. the wild-type strain. A total of 2743 genes were differentially expressed at least twofold level either between the parent and Δ*lae1* or the parent and *lae1*OE (at $P < 0.05$; Table 1; for complete list of genes see http://www.g3journal.org/content/3/2/369/suppl/DC1, Table S2). Only 71 genes were down-regulated in Δ*lae1* and up-regulated in *lae1*OE (**Table 1**), a pattern often observed for secondary metabolite cluster expression in other systems (Bok and Keller 2004, Georgianna et al. 2010). The majority of genes (1113) were unaffected in the Δ*lae*1 strain but upregulated in the *lae1*OE strain. A set of 372 genes were significantly down-regulated in both the Δ*lae1* and *lae1*OE strains. Interestingly, no transcripts were found that were up- or down-regulated in Δ*lae1* but unaffected in *lae1*OE. The remaining 1188 genes included 17 genes that were up-regulated in Δ*lae1* and *lae1*OE, 930 that were downregulated in the *lae1*OE strain but unaffected in Δ*lae1*, and 240 that were up-regulated in both mutant strains (see **Table 1** for details).

Table 1 Up-regulated and down-regulated genes in lae1 manipulated strains of *T. reesei*

Δ*lae1*	***lae1*OE**	**Genes**
Down	Up	71
Down	Down	372
Down	a	0
UP	Down	17
UP	a	0
UP	Up	240
a	Up	1113
a	Down	930
Total		2743

[a] indicates expression <two fold in either direction, and is therefore is considered to be unaffected

Of the 1556 genes whose transcriptional behavior was consistent with a positive action of LAE1 (indicated in bold in **Table 1**), 221 genes shared no orthologs with any other fungus for which data are available, and 588 encoded unknown proteins that were conserved in other Pezizomycota (analyses done on July 3, 2012). To identify the gene families that were significantly affected by either loss-of-function or overexpression of LAE1, we expressed them as percentage of the total number of transcripts in the respective fraction of the transcriptome (i.e., the categories shown in **Table 1**), and compared this with the percentage of these genes in the total genome (**Table 2**).

Table 2 Grouping of LAE1-dependent differentially expressed genes by functional domains

	No. in Genome	%	Down/ Up	%	N/Up	%	Down/ down	%
Total Number	9143		71		1113		372	
Ankyrins	21	0.22	0		4	0.35	6	1.6
Glycosyl hydrolases	200	2.2	27	38	28	2.5	13	3.4
Cytochrome P450	22	0.76	0		14	1.25	6	1.6
Glutathione-S-transferases	38	0.4	0		5	0.45	3	0.8
HET (heteroincompatibility genes)	22	0.24	2	2.8	2	0.17	1	0.26
Hydrophobins	8	0.08	0		5	0.44	0	
Iron uptake	12	0.13	0		4	0.36	2	0.53
Metabolism	1888	20.6	2	2.8	55	4.9	49	13.1
Mitochondrial biogenesis	186	2	0		12	1.1	0	
Transporters of the major facilitator superfamily	220	2.4	2	2.8	13	1.1	24	6.4
Multidrug transporters	26	0.28	0		7	0.63	4	1.1
Orphan genes	1012	11	7	9.8	71	6.3	25	6.7
Enzymes of O2 metabolism	75	0.82	2	2.8	12	1.1	11	2.9
PKS and NRPS	23	0.25	0		4	0.35	1	0.25
Proteases	383	4.1	1	1.4	22	1.9	4	1
PTH11a	24	0.26	1	1.4	6	1.05	1	0.25
Ribosome biogenesis	339	3.7	0		23	2	0	
Protein secretion	313	3.4	1	1.4	16	1.4	0	
Small secreted cysteine-rich proteins	174	1.9	3	4.2	52	4.6	3	0.8
Transcription factors	227	2.4	2	2.8	13	1.1	22	6.3
Unknown proteins	3535	38.7	12	16.9	445	40	111	22.8

The number of genes with polyadenylated transcripts that change in the *T. reesei* transcriptome when *lae1* expression is changed were compared with the total number of genes in the genome and ordered according to three expression patterns shown in Table 1. Percentage values printed in bold are significantly different to the percentage of these genes in the genome ($P < 0.05$); values were rounded to the nearest decimal.

[a] G-protein coupled receptors typified by PTH11, a cell-surface integral membrane protein required for pathogenicity in *Magnaporthe grisea* (Kulkarni et al. 2005).

This analysis revealed that the 71 genes with decreased expression in Δ*lae1* and up-regulated in *lae1*OE largely belonged to the glycosyl hydrolases reported recently (Seiboth et al. 2012); in addition, HET (heterokaryon incompatibility) genes, enzymes metabolizing molecular oxygen (FAD monooxygenases, FAD-dependent oxidases, catalases) and PTH11-type G-protein coupled receptors

(Kulkarni et al. 2005) were significantly abundant among these 71 genes. Genes that were unaffected in the Δ*lae1* strain but up-regulated in *lae1*OE included five of the eight class two class II hydrophobins of *T. reesei* (Druzhinina et al. 2012), genes for iron uptake and again PTH11 receptors. Genes encoding ankyrins [a 33-residue motif that mediates molecular recognition via protein–protein interactions and that has been shown to be involved in pathogenesis and endosymbiosis in bacteria (Breeuwer and Jacobs 1996)], enzymes involved in reactions with molecular, and also some other genes for iron uptake were abundant among those that were down-regulated in both Δ*lae1* and *lae1*OE (**Table 2**). The PTH11 and HET genes were chosen to validate the array data, indicating good correlation between array and quantitative PCR results [Table A1 (Appendix 2)].

lae1 overexpression has a greater impact than *lae1* loss on secondary metabolite gene expression in *T. reesei*

The *T. reesei* genome contains 10 genes for nonribosomal peptide synthases (NRPS), 11 polyketide synthase (PKS) genes, and two NRPS/PKS fusion genes (Martinez et al. 2008, Baker et al. 2012). In contrast to results from genome-wide expression analysis in *Aspergillus fumigatus* (Perrin et al. 2007), *A. flavus* (Georgianna et al. 2010), and *Fusarium verticillioides* (Butchko et al. 2012), that revealed decreased expression (60–80%) of PKS and NRPS genes in LaeA loss-of function mutants, a much smaller portion of these genes is affected in *T. reesei* by LAE1 perturbation. As shown in **Figure 1A**, 16 of these 23 (eight NRPS and eight PKS genes) were significantly expressed under the present conditions, but only seven of them [Trire2:105804, a nonreducing PKS of clade III; Trire2:65172, a reducing PKS of the lovastatin clade, and Trire2:65116, a reducing PKS of the fumonisin clade (Baker et al. 2012)] and a single NRPS (Trire2:24586, encoding one of the two siderophore synthases) were up-regulated in the *lae1*OE strain. Note that these four genes were essentially not expressed in the parent and the Δ*lae1* strain and thus may generate cryptic secondary metabolites (**Figure 1B**). In addition, the second siderophore synthase (Trire2:69946) was down-regulated in both mutants.

Furthermore, one NRPS [Trire2:23171 paracelsin synthase (Neuhof et al. 2007)] and one PKS [Trire2:59482; a reducing t-toxin like PKS from clade III (Baker et al. 2012)] were significantly up-regulated but in the *lae1*OE but also Δ*lae1* strain. None of the aforementioned PKS genes or their products has as yet been characterized. Other secondary metabolites and the genes involved in their synthesis pathways are not known from *T. reesei*. However, we noticed a high number of genes encoding cytochrome p450 monooxygenases, flavindependent monooxygenases, short chain dehydrogenase/reductases, and methyltransferases to be up-regulated in the *lae1*OE strain, and an approximately similar number of other genes for the same type of enzymes to be down-regulated in both mutant strains (**Figure 1C**, **Table 2**). These genes may be involved in secondary metabolite biosynthetic pathways.

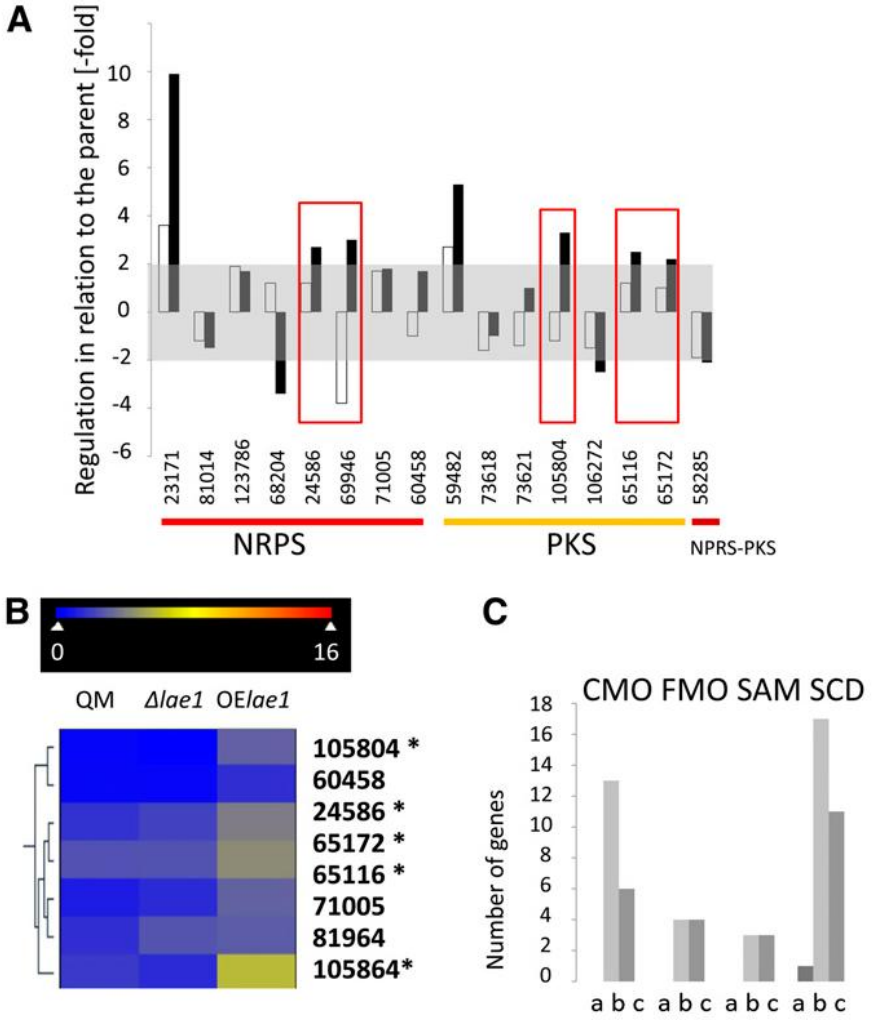

Fig. 1. Effect of modulation of *lae1* gene expression on the expression of secondary metabolism genes. (A) Ratios of increased or decreased expression in Δ*lae1* (white bars) and *lae1*OE (black bars), relative to the parent strain. The gray area indicates ± twofold, which is not considered significant. Genes are indicated by the Trire2 accession numbers (http://genome.jgi-psf.org/Trire2/Trire2.home.html) and are ordered as NRPS, PKS, and NRPS-PKS (indicated below the numbers). Only genes with P <0.05 are shown. The red boxes surround genes that are likely regulated by LAE1, as described in the text. (B) Excerpt of a hierarchical cluster analysis of PKS and NRPS gene expression showing a cluster that contains all the significantly regulated genes. Data are shown as a heat map, and the color code of respective expression values (dark blue: 0; dark red: 16; numbers indicate the $\log_2$ of the mean expression level, n = 2) (C) Number of genes putatively involved in secondary metabolism that occur in one of the groups significantly regulated by LAE1 (a, down-regulated in Δ*lae1* and up-regulated in *lae1*OE; b, unaffected in Δ*lae1* but up-regulated in *lae1*OE; c, down-regulated in both). CMO, cytocrome P450cytochrome p450 monooxygenases; FMO, FAD monooxygenases; SAM, S-adenosyl-methionine-dependent methyltransferases; and SCD, short-chain dehydrogenases/reductases.

Perturbation of LAE1 expression does not correlate with changes in histone H3 methylation patterns

LaeA has been proposed to counteract histone H3 lysine 9 trimethylation (H3K9me3) in the sterigmatocystin gene cluster of *A. nidulans* (Reyes-Dominguez et al. 2010). To begin to investigate whether LAE1 influences the lysine methylation status of H3 in *T. reesei*, we performed ChIP-seq with

antibodies against histone modifications known to be associated with transcriptionally active (H3K4me2 and -me3) or silent (H3K9me3) chromatin on the parental, the Δ*lae1* and the *lae1*OE strain. Under the conditions used, of the 9143 predicted genes in *T. reesei*, 4089 were significantly associaed with at least one of the three methylation marks in at least one of each of the three strains (http://www.g3journal.org/content/3/2/369/suppl/DC1, Table S4). Of these, only 993 of these 4089 genes (24%) showed significant regulation as measured by the perturbation of LAE1 (**Table 3**). To test the default hypothesis that LAE1 would counteract H3K9 methylation, we screened the 993 genes for those that are methylated at H3K9 in the Δ*lae1* strain but not in the parent and the *lae1*OE strain. Only three such genes were found (Trire2:66927, Trire2:53452, and Trire2:41942) and all encode unknown proteins. H3K9me3 constituted only a small portion of the LAE1-regulated genes (40 in total), and 31 of these were found to be methylated in all three strains. As for H3K4me2 and -me3, 557 genes showed both methylation marks in all three strains (WT, Δ*lae1*, and *lae1*OE) and were thus also LAE1-independent.

Table 3 Summary of H3K4 di- and trimethylation patterns for genes that are not associated with methylated H3K4 in Δlae1 but are associated with methylated H3K4 in at least one other strain

	H3K4me2			**H3K4me3**		**Regulation (Δ*lae1*/*lae1*OE)**				
WT	Δ*lae1*	*lae1*OE	WT	Δ*lae1*	*lae1*OE	Down/up	N/up	Down/down	N/down	Up/up
+		+	+		+		1	7	1	
+		+			+	1	1	1	2	
+		+				2	5	5	9	1
+			+		+			1		
+							1	5	3	
			+		+		6	13	12	1
		+	+		+	1				1
					+			2	2	11
		+			+	1				
			+				2	4	9	2
		+					10	6	8	11
			In total			4[a]	26	44	46	27

+ indicates the presence of methylation in this strain, no symbol indicates absence; numbers specify the number of genes that show the respective pattern [up or down before the slash refers to regulation in Δ*lae1* vs. parent strain (WT), whereas right of the slash refers to regulation in the *lae1*OE vs. WT strain; N indicates no significant regulation (<twofold in either direction)]. WT, wild type.
[a] *lae1* not included

We therefore screened the remaining 430 genes for those that would bear either di- or trimethylation, or both, on H3K4 in the parent and/or the *lae1*OE strain but not in the Δ*lae1* strain.

We found 148 genes that met these criteria (**Table 3**). Of these, only four genes showed the expected down-regulation in the Δ*lae1* strain and upregulation in the *lae1*OE strain if the same paradigm would hold for *T. reesei* as for *A. nidulans*. We found 26 genes that were up-regulated in the *lae1*OE strain only, and 44 genes that were down-regulated in both strains. There was no methylation pattern that clearly correlated with any of these three patterns of gene regulation. Annotation of these 74 genes showed that they comprised 17 genes of unknown function and six orphan genes known only from *T. reesei*, four genes encoding CAZymes, four genes encoding transcription factors, and three genes for permeases of the major facilitator superfamily [Table A2 (Appendix 2)]. None of the gene families that were enriched for LAE1 regulation (**Table 2**) was particularly abundant among these 74 genes. ChIP-seq results on selected targets were validated by region-specific PCR and results were similar, i.e., no enrichment differences for any of the histone marks in the three different strains in any of the regions tested [data not shown (Seiboth et al. 2012)].

Functional *T. reesei lae1* does not complement an *A. nidulans* Δ*laeA* strain

A phylogeny of LaeA/LAE1 amino acid sequences resulted in a tree that resembles the species tree, indicating that LAE1 is the true rtholog of LaeA (Seiboth et al. 2012) and suggesting that Lae1/LAE1 is conserved among fungi. To learn whether it is also functionally conserved, we transformed an *A. nidulans* Δ*laeA* mutant with the *T. reesei lae1* gene. Northern analysis showed that *lae1* was transcribed in the positive transformants (**Figure 2A**). Mycelial growth of *A. nidulans* was unaffected by *laeA* loss-of-function, and also the introduction of *T. reesei lae1* into the Δ*laeA* mutant had no effect (data not shown). Expression of *T. reesei lae1* in *A. nidulans* partially recovered the decrease in asexual sporulation in light but not in the dark (**Figure 2B**). Interestingly, *lae1* expression further decreases the formation of sexual spores (**Figure 2C**), which was correlated with a decrease in cleistothecia/unit area than both WT and D*laeA* (**Figure 2D**). Those cleistothecia that were formed

were abnormally large. In addition, *T. reesei lae1* was unable to restore sterigmatocystin production in *A.nidulans* (**Figure 2E**).

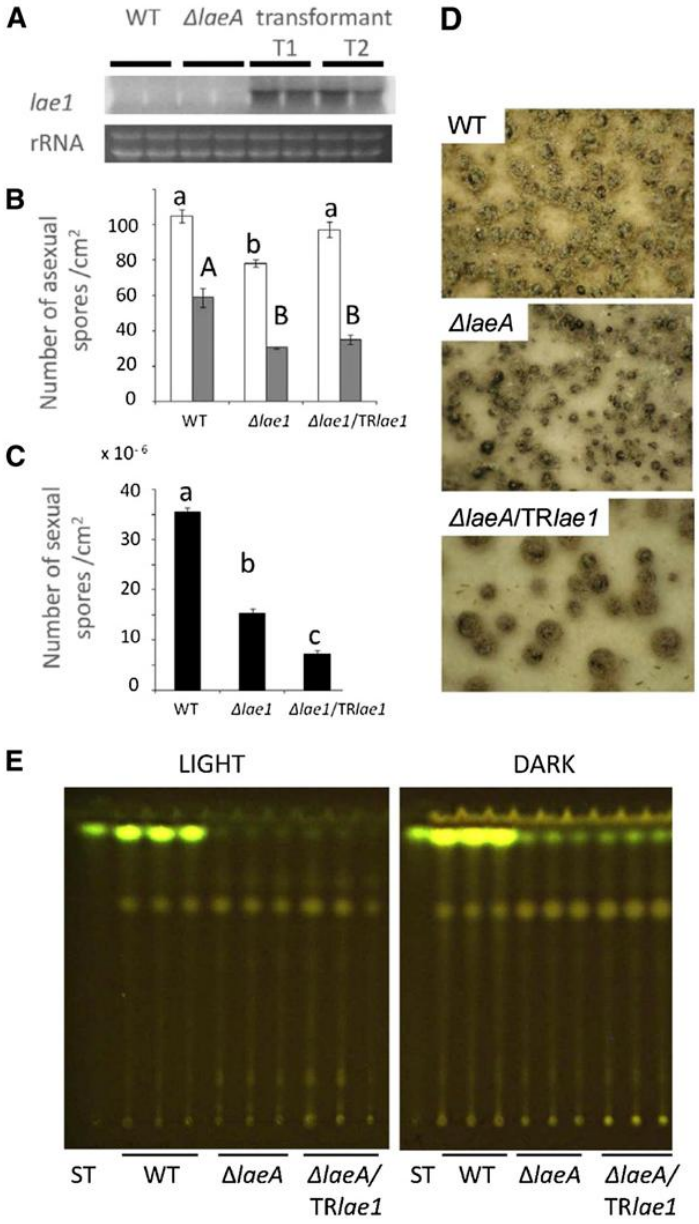

Fig. 2. Effects of introducing *T. reesei lae1* in *A. nidulans* D*laeA*. (A) Northern blot demonstrating expression of *Trlae1* mRNA in two *A. nidulans* transformants (T1, T2). (B) Overexpression of *lae1* leads to an impairment of asexual spore formation in a D*laeA* background. Light bars are strains grown in light, and dark bars are strains grown in dark. (C) Overexpression of *lae1* reinforces the impairment of sexual spore formation in a D*laeA* background. For both panels B and C, letters indicate statistically significant differences ($P < 0.05$) for each strain at different population levels according to the Tukey-Kramer multiple comparison test. Error bars show standard deviations of the results of three replications. (D) Sexual development of the WT, the D*laeA* mutant, and a D*laeA*/*TRlae1* strain on Champs medium in the dark for 5 d. (E) LAE1 does not complement sterigmatocystin production in a *laeA* mutant of *A. nidulans*. Wild-type (WT), the D*laeA* mutant, and a *lae1* transformant D*laeA*/*TRlae1* were grown on GMM solid media in either light or darkness for 5 d at 37° and extracts. Extracts were separated on a TLC plate. Extraction was triplicated. Each strain was extracted twice. Norsolorinic acid (orange) and sterigmatocystin (yellow) were visualized using long-wave (254 nm) UV light. A sterigmatocystin standard (ST) was spotted on left side of the plate. WT, wild type.

Taken together, these data suggest that LAE1 cannot complement an *A. nidulans* Δ*laeA* mutant and even appears to interfere with its sexual development.

T. reesei LAE1 does not interact with *A. nidulans* VeA

As outlined previously, LaeA is part of the heterotrimeric velvet complex, which is assembled in the nucleus in the dark and additionally contains the VeA (velvet) protein, a regulator of morphogenesis and secondary metabolism in some filamentous fungi (Bayram and Braus 2011), and the VeA-related developmental regulator VelB (velvet-like B). VelB interacts with the N-terminus of VeA, whereas LaeA interacts with the C-terminus of VeA (Bayram et al. 2008a). We therefore hypothesized that the inability of LAE1 to replace LaeA may be due to a lack of interaction of *T. reesei* LAE1 with *A. nidulans* VeA. To test this, we used a yeast two-hybrid system using the *T. reesei* and *A. nidulans* LAE1/LaeA proteins, respectively, as "bait," and the *T. reesei*/*A. nidulans* VEL1/VeA proteins as "prey." LaeA and VeA and LAE1 and VEL1 clearly interacted with each other, as did LaeA and VEL1 (**Figure 3**). However, in this assay *T. reesei* LAE1 was unable to interact with *A. nidulans* VeA. We therefore conclude that the inability of LAE1 to complement the Δ*laeA* strain may be caused by the inability of LAE1 to interact with the *A. nidulans* VeA protein.

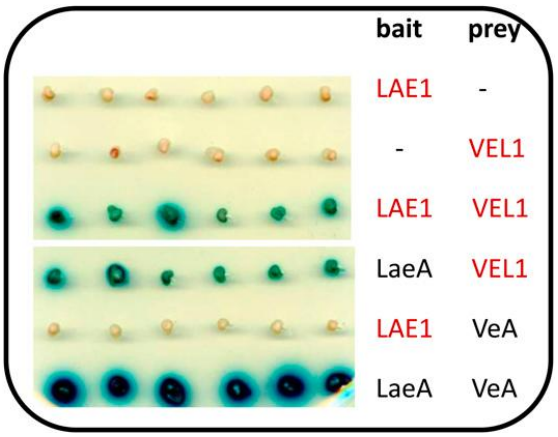

Fig. 3. Interactions among LaeA, VeA, LAE1, and VEL1 in a yeast two-hybrid assay. Derivatives of yeast strain L40 expressing the different bait and prey fusion proteins were selected on -UTL (-leu, -trp, -ura) containing 2% (w/v) glucose (SD) media. Six transformants of each combination were tested for their coloration on -UTL medium containing X-Gal for b-galactosidase activities. Trichoderma proteins are marked in red.

LAE1 is necessary for *vel1*—but not *vel2*—gene expression

In *A. nidulans*, expression of three velvet-like genes, *veA*, *velB*, and *vosA* are enhanced in a Δ*laeA* mutant (Bayram and Braus 2011). To test whether this regulation is conserved in *T. reesei*, we used quantitative PCR to quantify the expression of the *veA* ortholog *vel1* (Trire2: 122284) in both wild-

type and Δ*lae1* strains. In contrast to *A. nidulans*, we found the transcript of *vel1* to be strongly downregulated in Δ*lae1* (29% of the control, P = 0.043) and to be unaffected in *lae1*OE (103%; P = 0.022). Inspection of the normalized microarray data revealed no effect on the putative *vel2* (Trire2:40551), and *vos1* (Trire2:102737) orthologs of *velB* and *vosA*, respectively (differences, 15% in either strain, P < 0.05).

Protein kinase A negatively regulates *lae1* expression

Because the aforementioned regulatory influences are different from those detected in *A. nidulans*, we also tested whether the regulation of *lae1* gene expression was similar to that reported for in *A. nidulans* (Bok and Keller 2004). In the latter fungus, *laeA* was shown to be subject to inhibition by protein kinase A (PkaA), suggesting a potential role of cyclic adenosine monophosphate (AMP). We therefore investigated the *lae1* transcript abundance in *T. reesei* strains modulated in the catalytic subunit of protein kinase A (PKA1), adenylate cyclase, and the G-protein GNA3, using Δ*pka1* and Δ*acy1* strains (Schuster et al. 2012) and a strain bearing a constitutively activated allele of *gna3*, which causes accumulation of cyclic AMP (Schmoll et al. 2009). Loss of function in PKA1 strongly enhances *lae1* transcript abundance (5.2 [± 1.3]-fold; P < 0.05), indicating similar regulation as found in *A. nidulans*. However, neither the adenylate cyclase mutant Δ*acy1* nor the constitutively activated *gna3* allele showed a significant effect (0.7 [± 0.5]-fold, and 1.8 [± 0.9]-fold; respectively). We conclude that PKA1 is a negative regulator of *lae1* expression in *T. reesei*, whereas the role of cyclic-AMP is unclear. Opposite effects of Δ*pka1* and Δ*acy1* on expression of cellulase genes in *T. reesei* have been noted earlier (Schuster et al. 2012).

Discussion

T. reesei lae1 was originally identified by a phylogenetic approach (Seiboth et al. 2012), and by the synteny of its chromosomal locus in *Fusarium* and *Trichoderma* (Christian P. Kubicek, unpublished

data). The latter approach was useful as many filamentous fungi express several LaeA homologs (Christian P. Kubicek, unpublished data, Jiang et al. 2011). We consider this important because some of the present results, such as lack of complementation of *A. nidulans*, and the small effect of *lae1* knock out on PKS and NRPS gene expression, however, may cast doubt on the identity of LAE1 as a true ortholog of the LaeA proteins characterized from other fungi, e.g., *A. nidulans*, *A. fumigatus*, *A. flavus*, *P. chrysogenum*, *F. fujikuroi*, *F. verticillioides*, and *C. heterostrophus* (Bok and Keller 2004, Bok et al. 2005, Amaike and Keller 2009, Kosalková et al. 2009, Hoff et al. 2010, Wiemann et al. 2010, Butchko et al. 2012, Wu et al. 2012). In fact, besides the loss of or enhancement of sporulation in the Δ*lae1* and *lae1*OE strains (Seiboth et al. 2012), respectively, which is largely conserved in many of the aforementioned taxa, and the demonstration of interaction with LAE1 with *T. reesei* VEL1, other findings seem to differ from those obtained with other fungi.

One of the most striking differences was the inability of *lae1* to complement an *A. nidulans* Δ*laeA* strain. Because *laeA* was well expressed in *A. nidulans*, this could be due to incorrect translation or subtle differences in the aa structure of LaeA and LAE1. The interaction studies demonstrate that this may be due to the lack of ability of LAE1 to interact with VeA, and effects observed by LAE1 in the *A. nidulans* Δ*laeA*/*Trlae1* transformants may thus be interpreted as being caused by an excess of unbound LAE1. This finding was puzzling because complementation of *A. nidulans* Δ*laeA* was successful with Lae1 from *F. fujikuroi* (Wiemann et al. 2011), which is phylogenetically closer to *T. reesei* than to *A. nidulans*. An alignment of the *T. reesei* LAE1 protein with that of other published LaeA/LAE1 proteins (**Figure 4**) shows an overall amino acid (aa) identity of 70% with *Fusarium spp*. Since the N-terminus of LaeA has been demonstrated to be involved in binding to VeA (Bayram et al. 2008a) differences in its structure could interfere with this binding. However, the N-terminus is generally poorly conserved between fungi, and we could not detect specific differences that would offer an explanation for the lack of binding between VeA and LAE1.

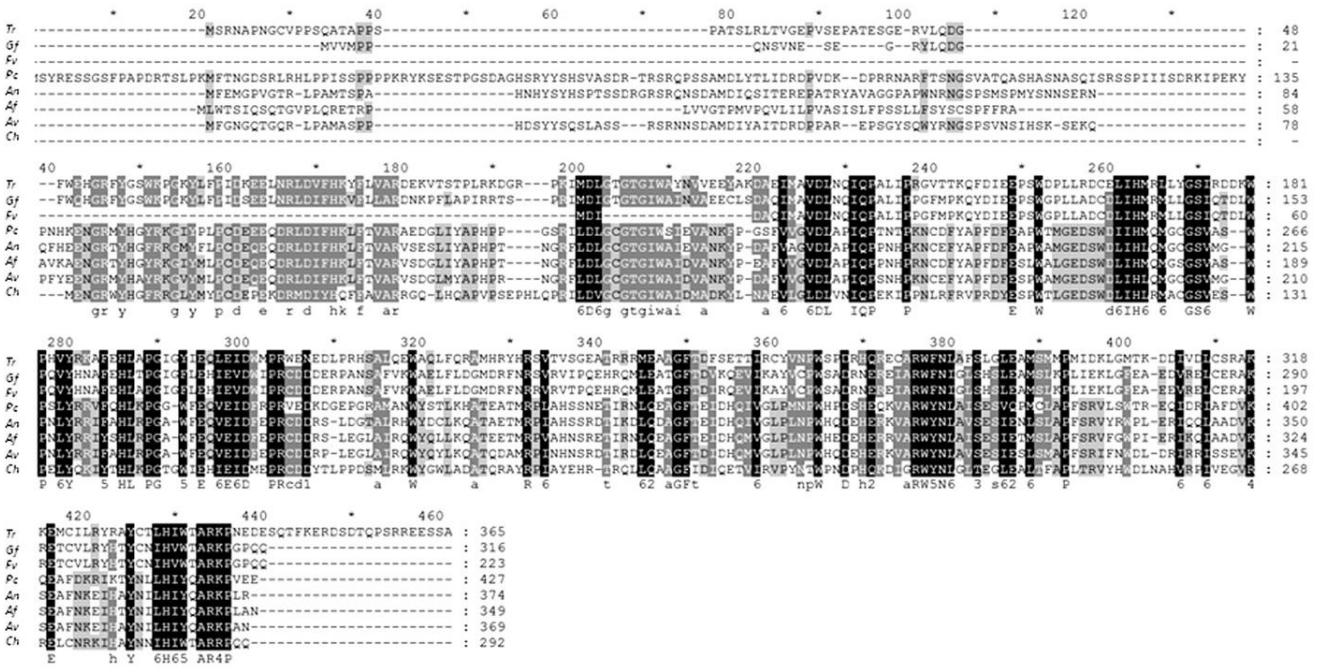

Fig. 4. Alignment of published LaeA/LAE1 sequences. Abbreviations and accession numbers: *Tr*, *T. reesei* AFK30952.1; *Gf, G. fujikuroi* CBE54370.1; *Fv, Fusarium verticillioides* FVEG_00539 (not deposited; sequence taken from http://www.broadinstitute.org/annotation/genome/fusarium_group/MultiHome.html); *Pc*, *P. chrysogenum* ACD50375.1; *An*, *A. nidulans* AAQ95166.1; Af, A. fumigatus AAR01218.1; Av, A. flavus AAX68412.1; and *Ch*, *C. heterostrophus* AEP40318.1.

Another possible explanation of the impact of *lae1* on *A. nidulans* - as well as its function in *T. reesei*- could be related along lines of those observed for another recently described *laeA*-like putative methyltransferase, called LlmF, in *A. nidulans* (Palmer et al. 2012). Using a bioinformatics approach, 10 predicted methyltransferase proteins with similarity to LaeA were characterized in *A. nidulans* with regard development and secondary metabolism. One of these proteins, LlmF, was found to exhibit an opposite phenotype to *laeA* where overexpression of *llmF* decreased secondary metabolism and sexual development and deletion of *llmF* increased secondary metabolism. LlmF was found to redirect cellular localization of VeA, thus resulting in the aforementioned phenotypes. Possibly LAE1 could have some impact on VeA function in *A. nidulans* despite negative yeast two-hybrid results. It is thus possible that other *lae1*-like proteins in *T. reesei* may impact expression of secondary metabolite gene clusters. There is also precedence for this notion from some preliminary studies on “velvet-interacting proteins” in *F. graminearum* (Jiang et al. 2011).

LaeA has been identified as a global transcriptional regulator of secondary metabolism in *A. nidulans* (Bok and Keller 2004) and subsequent work in other Aspergilli (Bok et al. 2005, Georgiianna et al. 2010), *P. chrysogenum* (Hoff et al. 2010), *F. fujikuroi* (Wiemann et al. 2010), *F. verticillioides* (Butchko et al. 2012), and *C. heterostrophus* (Wu et al. 2012) has lend credence to this idea. In *A. fumigatus*, expression of 13 of the 22 of the gene clusters for secondary metabolite synthesis was significantly down-regulated in D*laeA* (Perrin et al. 2007). An even stronger effect was observed in *F. verticillioides*, where the transcription of 14 of 16 PKS genes was affected in a *lae1* mutant (Butchko et al. 2012). In *T. reesei*, in contrast, only five of the total 23 PKS and NRPS genes were affected in the Δ*lae1* strain. Although we cannot exclude that the conditions for growth of *T. reesei* in this study were not those that best favor secondary metabolite gene expression, 10 of the 23 genes were at least moderately expressed in the parental strain. Interestingly, the seven genes that were up-regulated in the *lae1*OE strain were all characterized by a very low expression level in the parent strain, which would be compatible with assuming that overexpression of LAE1 may activate silent genes (Bok et al. 2006b). Nevertheless, the lower number of PKS and NRPS genes that are affected by LAE1, compared with other fungi, may suggest that regulation of their biosynthesis is not a major target of LAE1 *in T. reesei*. In *A. fumigatus*, an additional 38% of all cytochrome p450 monooxygenase genes also showed differential expression in D*laeA*, and 15 of them were located in secondary metabolite clusters (Perrin et al. 2007). This compares well to *T. reesei*, where we detected LAE1-dependent expression of 20 from a total of 70 cytochrome P450 monooxygenases. However, it is unknown whether they are indeed involved in secondary metabolite synthesis in *T. reesei* or fulfill other functions such as detoxification (Druzhinina et al. 2012). Only six cytochrome P450 monooxygenases were found among the 203 genes downregulated in the Δ*lae1* mutant of *F. verticillioides* (Butchko et al. 2012). The precise molecular function of LaeA/LAE1 and its mechanism of action remain enigmatic. Complex formation with VeA and VeB has been demonstrated, but its precise contribution to the regulation of secondary metabolism gene clusters is not yet known, even

in *Aspergillus* spp. In *A. nidulans*, LaeA somehow counteracts trimethylation of H3K9 and binding of the homolog of Heterochromatin Protein 1 (HP1/HepA) to this repressive chromatin mark (Strauss and Reyes- Dominguez 2011). Based on results presented here, this model is unlikely to be true for *T. reesei* as among a total of 1021 genes responding to modulation of *lae1* function, H3K9me3 was absent from most genes in the Δ*lae1* strain, and the methylation pattern of the 13 genes that indeed exhibited H3K9 methylation was independent of LAE1. Absence of H3K9me3 from chromatin associated with bona fide genes is also the default state in *N. crassa*, where heterochromatic histone modification marks are almost exclusively associated with transposon relics (Tamaru and Selker 2001, Tamaru et al. 2003, Lewis et al. 2009, Smith et al. 2011). Conversely, only 74 genes exhibited H3K4 methylation patterns that would be consistent with a role of LAE1 in H3K4 methylation, and the FunCat composition of the protein encoded by this gene sample was random. Based on our data, we conclude that LAE1 is not involved in the methylation of H3K4 or H3K9 in *T. reesei*. Currently, the evidence for direct histone methylation by LaeA/LAE1 is at best sparse. No true substrate has been identified yet. None of the LaeA homologs share domains found in many protein methyltransferases that specifically act on histones, e.g., SET domains found in Clr4/DIM-5/Suvar3-9, or motifs found in Dot1 (Sawada et al. 2004; Adhvaryu et al. 2005). LaeA/LAE1 shares some short regions of similarity with arginine methyltransferases. Arginine methylation of histone tails can promote or prevent the docking of transcriptional effector molecules and in this way influence the expression of gene clusters and regulons (Di Lorenzo and Bedford 2011). Two equally likely scenarios may involve the use of LaeA/LAE1 as a shuttle for methyl groups to other proteins in the velvet complex, based on the requirement of the SAM binding motif in Aspergillus LaeA (Bok et al. 2006a, Bayram and Braus 2011), or the possibility that LaeA/LAE1 methylate proteins in the velvet complex. However, we also cannot rule out other possible indirect effects of LAE1 such as on acetylation or on general nucleosome arrangements known to impact gene expression in fungi. For example, the histone acetyltransferase EsaA was recently shown to activate secondary metabolite gene clusters through H4K12 acetylation

in *A. nidulans* (Soukup et al. 2012). LaeA, in combination with the velvet family of related regulatory proteins, may be involved in supporting the development of progeny in Aspergilli, by controlling the production of chemicals to protect fruiting bodies and the production of nourishing cells for developing fruiting bodies (Sarikaya Bayram et al. 2010). Based on our current findings, we adapt this interpretation for *T. reesei*. This species is unique in the genus because it has escaped its evolutionary history as a mycoparasite to become a successful competitor in the use of predegraded wood (Druzhinina et al. 2011). Obviously, this lifestyle profits more from an efficient arsenal of (hemi)cellulases and proteases, and transporters for the respective hydrolysis products, than from excessive toxification of its competitors. In this study we identified LAE1-dependent expression of PTH11 G-protein-coupled receptors, heterokaryon incompatibility proteins, hydrophobins, iron uptake, and unknown proteins bearing ankyrin motifs (see **Table 2**). Regulation of iron uptake and hydrophobin formation by LaeA has also been detected in *A. fumigatus* (Perrin et al. 2007) and *A. flavus* (Georgianna et al. 2008, Chang et al. 2012), and genes encoding enzymes reacting with molecular oxygen are also present in the LaeA/LAE1-dependent transcriptomes of *A. fumigatus*, *A. flavus*, and *F. verticillioides* (Perrin et al. 2007, Georgianna et al. 2008, Butchko et al. 2012) but all the other gene categories are a specific response of *T. reesei*. As all these genes function in the response of the fungus to environmental stimuli and the presence of competing organism, we interpret this such that LAE1 is involved in successful establishment of *T. reesei* in its environment.

Materials and Methods

Strains and growth conditions Strains *T. reesei* QM 9414 (ATCC 26921), an early cellulase producing mutant, and the mutant strains C.P.K. 3793 (Δ*lae1*) and C.P.K. 4087 (*lae1*OE) derived from it (Seiboth et al. 2012), $gna3_{QL}$ (Schmoll et al. 2009), Δ*pkac*1, and Δ*acy1* (Schuster et al. 2012) were used throughout this work. They were grown in Mandels-Andreotti medium (Mandels & Andreotti 1978), using 1% (w/v) lactose as a carbon source.

*A. nidulan*s strains used or created in this study are RJW33.2 (Δ*laeA*::*metG*, *wA3*, *trp*801, *pyro*A4, *veA1*), TJW123.20 (Δ*laeA*::*metG*, *wA3*, *trp*801, *lae*1::*pyroA*, *veA1*), RDIT2.1 (*metG1*, *veA*), RJW41.A (Δ*laeA*::*metG*, *veA*), RDIT9.32 (*veA*), and RCSR4.16 (Δ*laeA*::*metG*, *lae1*::*pyroA*, *veA*). Unless otherwise noted, they were grown on glucose minimal medium, or GMM (Shimizu and Keller 2001), with additional supplements for auxotrophic strains (pyrodoxin, methionine or tryptophan). All strains are maintained as glycerol stocks at -80 °C. *Escherichia coli* JM109 (Promega, Madison, WI) was used for plasmid construction and amplification.

Transformation of *A. nidulans* with *T. reesei lae1*

A *lae1* fragment obtained from pRKA41617ptrA (containing a 2.8-kb genomic clone of the *T. reesei lae1* that contained 900 bp of 5´ and 500 bp of 3´ noncoding regions) was inserted into *PstI* and *SpeI* sites of pJW53 to create pJW139.3 (Bok and Keller 2004), which places genes at the *pyroA* locus in *A. nidulans*. The recipient strain RJW33.2 was transformed with pJW139.3 as described previously (Bok and Keller 2004). Transformants were confirmed by polymerase chain reaction (PCR) and Southern blots and one correct transformant (TJW123.20) was sexually crossed with RDIT2.1 to create a prototrophic strain (RCSR4.16). Northern analysis was performed to assess *lae1* expression.

Nucleic acid analysis

The extraction of DNA from fungi and bacteria, restriction enzyme digestion, gel electrophoresis, blotting, hybridization, and probe preparation were performed by standard methods (Ausubel et al. 1999). Total RNA was extracted from *Aspergillus* strains by use of Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA blots were hybridized with ^{32}P-labeled *lae1* DNA fragments, which were generated by PCR using gene-specific primers, lae1SpeI (5´TACTAGTCTACCTCTTTCAAGGAGC) and lae1PstI (5´TCTGCAGACGAGAGATCATATATCCG).

Analysis of *A. nidulans* growth and development

Conidia (asexual spores) and ascospore (sexual spores) production were examined using point inoculation or overlay methods in light and/or dark conditions. In point inoculation, 1 µL of 10^6 conidia/mL was deposited on the middle of the plate. In overlay, 5 mL of top medium (0.75% agar) with 10^6 conidia was applied onto the bottom medium. Point inoculated colonies were cultured by inoculating on GMM plates with 10^3 spores and incubating in light and dark conditions at 37 °C for 5 d. Colony diameter was measured and 1-cm cores from the center were taken and homogenized with 3 mL of water. Conidia were counted using a hemocytometer under a light microscope. Conidia per milliliter were determined by standard calculation (counted conidia in total grid Xx dilution factor X 10^4). For overlay cultures, 10^6 spores in 5 mL of CHAMPS medium (0.75% agar) (Greene et al. 2003) were overlaid on top of 25 mL of CHAMPS medium (20 g of glucose, 16 g of agar, 5 g of yeast, and 1 mL of trace element solution per liter of medium) and then incubated in the dark to promote sexual development at 37 °C for 5 d. One-cm cores were taken from the plates, homogenized in 3 mL of water, and released ascospores were counted using a hemocytometer under a light microscope.

Statistical analysis

Spore data were statistically compared by analysis of variance using the Tukey-Kramer test for multiple comparison. Statistically significant mean values, indicated with different letters in the figures, are significant at $P < 0.05$.

Metabolite assays

Thin-layer chromatography (TLC) was used to assess sterigmatocystin production. One-centimeter cores were punched from the center of point inoculated plates and homogenized with 3 mL of sterile doubledistilled H_2O. Three milliliters of chloroform were added, mixed well, and the samples centrifuged for 10 min. The organic layer was removed, transferred into a 3-mL glass vial, and left to

dry in a fume hood overnight. Dried extracts were resuspended with 100 mL of chloroform and 5 or 10 mL were loaded onto a non-ultraviolet (UV)-coated TLC plate. Sterigmatocystin was spotted as a standard. The plates were run in chloroform/acetone (8:2) solvent and stained with 15% aluminum chloride in 95% ethanol. TLC plates were viewed under 254-nm UV light.

Yeast two-hybrid analysis

The yeast two-hybrid plasmids pTLex3 and pGAD424 (Cho et al. 2003) were modified by the "quick change" technique (van den Ent and Löwe 2006) using *Pfu*Ultra II fusion HS DNA polymerase (Stratagene, Santa Clara, CA). The bait plasmid with *lae1* cDNA was constructed using two quick change primers, TlaeALexAFWD (5´CGCAACGGCGACTGGCTGGAATTCAAGCTTA TGTCTCGAAACGCTCCCAACGGGTGTG) and TlaeALexAREV2 (5´CTTGGCTGCAGGTCGACTCGAGCGGCCGTTAAGCAGAGGATTCCTCT CTTCTAGATGGC) to place *lae1* into pTLex3. *lae*1-pTLex3 was cotransformed into the Saccharomyces cerevisiae reporter strain L40 with either pGAD424 (empty prey vector) or with A. *nidulans veA* cDNA in pGAD424 (Bayram et al. 2008b). The prey plasmid with *vel1* cDNA was constructed using two Quick change primers, TveA424FWD (5´GAGATCGAATTCCCGGGGATCCGTCGAATGGCGACGCCTTCCTCCGTGGCCTCGTC) and TveA424REV (5´GCACAGTTGAAGTGAAC TTGCGGGGTTTTTACACCTGGTATTGGTTGAAGGTGACAACG to place *vel1* into pGAD424. *ve1*-pGAD424 was cotransformed into the *S. cerevisiae* reporter strain L40 with either pTLex3 (empty bait vector), with *A. nidulans laeA* cDNA in pTLex3 (Bayram et al. 2008b) or with *lae1*-pTLex3. Also, *A. nidulans laeA* cDNA in pTLex3 was cotransformed with *A. nidulans veA* cDNA in pGAD424 as a positive control. Transformants were selected on -UTL (-ura, -trp, -leu) containing 2% (w/v) glucose (SD) media. Six transformants of each combination were tested for their coloration on -UTL medium containing X-Gal.

Transcriptome analysis

Mycelia of *T. reesei* were harvested after 26 hr of growth; total RNA extracted (Chirgwin et al. 1979), and purified using the RNeasy MinElute Cleanup Kit (QIAGEN, Hilden, Germany). RNA quality and quantity were determined on gels and using a Nanodrop spectrophotometer. High-quality purified RNAs were submitted to Roche-NimbleGene (40 mg per 3-microarray set) where cDNAs were synthesized, amplified, and labeled and used for subsequent hybridization.

A high-density oligonucleotide microarray (Roche-NimbleGen, Inc., Madison, WI) was constructed, using 60-mer probes representing the 9143 genes of *T. reesei*. Microarray scanning, data acquisition and identification of probe sets showing significant differences (at $P < 0.05$) in expression levels between different conditions were performed by Roche-NimbleGen (www.nimblegen.com). Values were normalized by quantile normalization (Bolstad et al. 2003) and the RMA algorithm (Irizarry et al. 2003). After elimination of transcripts that exhibited an SD >20% of the mean value within replicates, false discovery rates (Benjamini and Hochberg 1995) were used to assess the significance of values. Transcripts showing significantly different expression compared with the 18-hr control (at least twofold changes at $P < 0.05$) were grouped by k-means clustering as implemented in Array Star 3.0.1 (Array Star Inc., Madison, WI). Gene accession numbers were annotated according to version 2 of the *T. reesei* genome assembly (http://genome.jgi-psf.org/Trire2/Trire2.home.html), and ambiguous cases annotated manually. Genes were classified according to their major annotation in the MIPS Functional Catalogue (FUNCAT) (Ruepp et al. 2004). To determine whether there were differences in the functional categories in each cluster, the distribution of categories within each cluster was compared with the total distribution of the same cluster within all the annotated genes using independent x^2 tests. The microarray data and the related protocols are available at the GEO web site (www.ncbi. nlm.nih.gov/geo/) under accession number GSE22687 (platform GPL10642).

Chromatin immunoprecipitation (ChIP) and ChIP-sequencing

To carry out ChIP-sequencing with *T. reesei*, we adapted a protocol developed for *Neurospora crassa* (Tamaru et al. 2003, Smith et al. 2011). QM 9414, Δ*lae1*, and *tef1::lae1* strains were grown for 5 d in the dark on 2% PDA medium and spores harvested. Flasks with 50 mL of lactose medium were inoculated with either 1 X 10^5 or 1 X 10^6 spores 21 mL $^{-1}$ and grown in the dark for 26 hr. All further steps were as described previously (Tamaru et al. 2003). DNA obtained by ChIP was suspended in 30 mL and used for construction of ChIP-seq libraries (Pomraning et al. 2009, Pomraning et al. 2012). We obtained 1.4–4.8 million mapped reads (between 76% and 98% of the total reads) for the nine libraries we sequenced (three strains and three antibodies). The antibodies used were from Active Motif (H3K4me3, 39159; H3K9me3, 39161) and Millipore (H3K4me2, 07-030). We used one additional H3K9me3 antibody from Abcam (ab8898), which resulted in less enrichment than with the Active Motif antibody (data not shown).

Real-time PCR

DNase I-treated (Fermentas) RNA (5 mg) was reverse-transcribed with the RevertAid First Strand cDNA Kit (Fermentas) according to the manufacturer's protocol with a combination (1:1) of the provided oligo-dT and random hexamer primers. All real-time PCR experiments were performed on a Bio-Rad (Hercules, CA) iCycler IQ. For the reaction the IQ SYBR Green Supermix (Bio-Rad) was prepared for 25-mL assays with standard $MgCl_2$ concentration (3 mM) and a final primer concentration of 100 nM each. All assays were carried out in 96-well plates which were covered with optical tape. Primers, amplification efficiency and R-square values are given in Supporting Information, [Table A3, (Appendix 2)]. Measurements for *tef1* were performed with both protocols for reference calculation. Determination of the PCR efficiency was performed using triplicate reactions from a dilution series of cDNA (1; 0.1; 0.01; 0.001). Amplification efficiency was then calculated from the given slopes in the IQ5 Optical system Software v2.0. Expression ratios were

calculated using REST Software (Pfaffl et al. 2002). All samples were analyzed in two independent experiments with three replicates in each run.

Acknowledgments

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Chapter 5

The Putative Protein Methyltransferase LAE1 of *Trichoderma atroviride* is a Key Regulator of Asexual Development and Mycoparasitism

The results presented in this chapter have been accepted in PLos ONE.
Razieh Karimi-Aghcheh, Irina Druzhinina, and Christian P. Kubicek
T. atroviride LAE1 is essential for asexual reproduction in the dark and for defense and parasitism on other fungi.

Abstract

In Ascomycota the protein methyltransferase LaeA is a global regulator that affects the expression of secondary metabolite gene clusters, and controls sexual and asexual development. The common mycoparasitic fungus *Trichoderma atroviride* is one of the most widely studied agents of biological control of plant-pathogenic fungi that also serves as a model for the research on regulation of asexual sporulation (conidiation) by environmental stimuli such as light and/or mechanical injury. In order to learn the possible involvement of LAE1 in these two traits, we assessed the effect of deletion and overexpression of *lae1* gene on conidiation and mycoparasitic interaction. In the presence of light, conidiation was 50 % decreased in a Δ*lae1* and 30-50 % increased in *lae1*-overexpressing (OE*lae1*) strains. In darkness, Δ*lae1* strains did not sporulate, and the OE*lae1* strains produced as much spores as the parent strain. Loss-of-function of *lae1* also abolished sporulation triggered by mechanical injury of the mycelia. Deletion of *lae1* also increased the sensitivity of *T. atroviride* to oxidative stress, abolished its ability to defend against other fungi and led to a loss of mycoparasitic behaviour, whereas the *OElae1* strains displayed enhanced mycoparasitic vigor. The loss of mycoparasitic activity in the Δ*lae1* strain correlated with a significant underexpressionn of several genes normally upregulated during mycoparasitic interaction (proteases, GH16 ß-glucanases, polyketide synthases and small cystein-rich secreted proteins), which in turn was reflected in the partial reduction of formation of fungicidal water soluble metabolites and volatile compounds. Our study shows *T. atroviride* LAE1 is essential for asexual reproduction in the dark and for defense and parasitism on other fungi.

Introduction

Comparison of the genomic inventory of *T. reesei*, *T. atroviride* and *T. virens* identified mycotrophy (i.e. successful feeding on either living or killed fungi) as the innate nature of the genus (Kubicek et al. 2011). This lifestyle involves a combination of traits such as host recognition, attachment to and

sometimes coiling around the host hyphae, and the secretion of antibiotic metabolites and cell-wall-degrading enzymes (Kubicek et al. 2011, Harman et al. 2004). The molecular mechanisms involved have been studied mainly with regards to the possible involvement of hydrolytic enzymes (chitinases, glucanases and proteases) and secondary metabolites (gliotoxin, peptaibols, 6-pentyl-2H-pyran-2-one [6PP]) in antagonism, and of heterotrimeric G proteins and their receptors in sensing of host signals (Omann et al. 2010). Mukherjee and Kenerley (2010) reported the developmental regulator VEL1 (an orthologue of the *Aspergillus nidulans veA*, which encodes a conserved global regulator of development and secondary metabolism; Calvo 2008, Bayram and Braus 2012) regulates mycoparasitism that in *T. virens*.

In *A. nidulans*, most of the effects of VeA depend on the formation of a trimeric protein complex consisting of VeA, VelB and LaeA (Bayram et al. 2008). The latter protein, a putative *S*-adenosylmethionine-dependent methyltransferase, was originally described as a global regulator of secondary metabolism in several *Aspergillus* spp. (Bok and Keller 2004, Bok et al. 2009), and later on shown to be also required for the biosynthesis of secondary metabolites in the industrially applied fungus *Penicillium chrysogenum* (e.g. penicillin) and the phytopathogenic fungi *Fusarium fujikuroi, F. verticillioides and Cochliobolus heterostrophus*, respectively (Hoff et al. 2010, Wiemann et al. 2010, Wu et al. 2012, Butchko et al. 2012). Further evidence emerged that LaeA also controls numerous developmental events in fungi, such as conidiation and fruiting body formation (Hoff et al. 2010, Wiemann et al. 2010, Wu et al. 2012). In plant and human pathogenic fungi, LaeA has also been demonstrated to be a virulence factor (Wiemann et al. 2010, Wu et al. 2012, Bok et al. 2005, Sugui et al. 2007).

We have recently studied the function of LAE1, the LaeA orthologue of *Trichoderma reesei* (Seiboth Karimi et al. 2012, Karimi-Aghcheh et al. 2013). Interestingly, in this fungus that has specialized to saprotrophic growth on pre-decayed wood, LAE1 is a major regulator for the expression of cellulases and hemicellulases that are required for feeding on this substrate (Seiboth Karimi et al. 2012, Karimi-

Aghcheh et al. 2013). One may thus hypothesize that LAE1 controls different strategies to aid the fitness of the fungus in its environment.

As emphasized above, mycotrophy is the innate nature of *T. atroviride* (Kubicek et al. 2011). In this work we have therefore tested the hypothesis that in *T. atroviride* LAE1 may be involved in mycoparasitic interaction of this species.

Results

Identification of the LAE1 orthologue of *T. atroviride*

We have recently identified LAE1 from *T. reesei*, and shown that it forms a supported clade with putative orthologues of *T. virens* and *T. atroviride* (Seiboth Karimi et al. 2012). The annotation of the *T. atroviride* orthologue (Triat2:302782; old number Triat1:42103) was manually corrected (correct sequence deposited under NCBI GeneBank accession number KC174792). LAE1 is encoded by a 1328 nt ORF that is interrupted by 5 introns and encodes a putative 363 aa protein. The aa's 110-205 specify the expected SAM-dependent methyltransferase domain (Pfam group PF13489).

Phenotype of *T. atroviride* LAE1 mutants

To investigate the function of *T. atroviride lae1*, we prepared knock out- and overexpressing strains (see Materials and Methods). Several mitotically stable Δ*lae1* and *OElae1* strains were obtained and verified by PCR (**Figure 1**). Several *Δlae1* strains and *OElae1* strains bearing one additional copy were then investigated with respect to growth and conidiation. Strains bearing the same mutation (Δ*lae1* or *OElae1* respectively; see below) displayed identical phenotypes. Despite several attempts we failed to introduce the wild-type *lae1* copy into the Δ*lae1* strains. Two Δ*lae1* or *OElae1* strains were thus selected for all further experiments, and gave essentially similar results. The Δ*lae1* strains showed a 25-30 % reduced growth rate on plates with D-glucose or D-galactose as a carbon source,

whereas growth of the *OElae1* strain was the same as that of the parent strain (data not shown). Otherwise the mutants did not show any morphological differences from the parent strain.

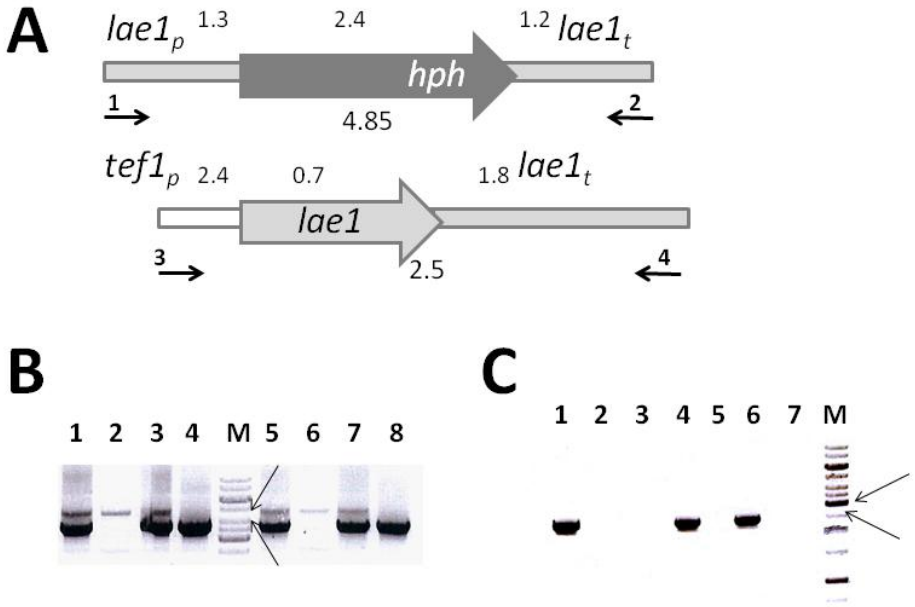


Fig. 1. Construction and proof for *T. atroviride OElae1* and *Δlae1* strains: (A) constructs used to disrupt *lae1* (top) and to express it under the *tef1* promoter (bottom). Numbers over the scheme indicate the size (in bp's) of the promoter, ORF and terminator used; the number below the scheme of the nucleotide fragment amplified by the respective primers used. Bold numbers over the small bold arrows specify the primers used: 1, Patro_FW_ConMeth_ApaI; 2, Tatro_Rev_ConMeth_SmaI; 3, tef1SC; 4, TrLae1TermHind. For primer sequences see Materials and Methods. (B) Identification of two *Δlae1* strains among 8 transformants (1-8); the two arrows point to the 5 and 4 kb marker (M) band (from top); (C) Identification of OE*lae1* strains among 6 transformants and the P1 parent strain (track 7). The two arrows point to the 3 and 2.5 kb marker (M) band

LAE1 is essential for cellulase formation in *T. atroviride*

In *T. reesei lae1* is essential for growth on cellulose and expression of cellulase and hemicellulase genes in *T. reesei* (Seiboth Karimi et al. 2012). In order to learn whether this is also the case in *T. atroviride,* we grew the strains on plates with carboxymethyl cellulose as the only carbon source and analyzed cellulase secretion by Congo red staining. Indeed, *T. atroviride* P1 exhibited a ratio of halo diameter vs colony diameter of 1.29 (±0.011), which was slightly enhanced in the *OElae1* strain (1.47 ±0.036). The Δ*lae1* strain, however, yielded a value close to 1 (1.07 ±0.016) indicating no or only very little cellulase secretion (which was also reflected in the very small colonies). Thus the observed LAE1-dependent regulation of cellulase formation in *T. reesei* also extends to *T. atroviride*.

LAE1 effects conidiation in *T. atroviride* in a carbon source and light/darkness dependent manner

The loss-of-function of *lae1* of *T. atroviride* significantly affected the intensity of conidiation, albeit the effect differed in light and darkness (**Figure 2**): when cultivated on PDA in light, conidiation intensity was reduced by approximately 50 % in the *Δlae1* strain. Conidiation in the dark, however, was reduced to almost zero. The *OElae1* strains, on the other hand, exhibited an increased conidiation density under illumination, whereas it displayed the same level of conidiation as the parent strain P1 in darkness (**Figure 2**).

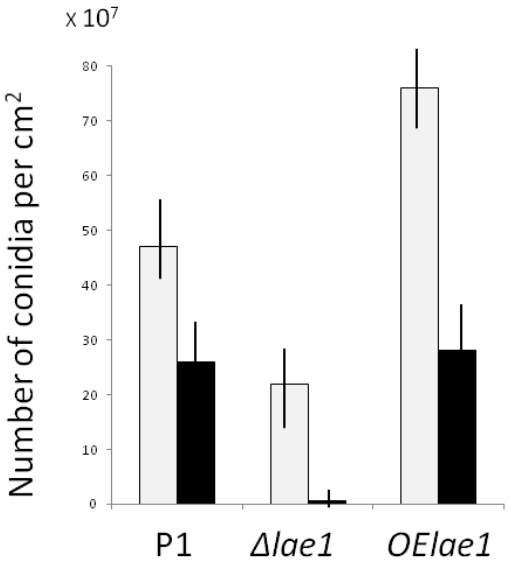


Fig. 2. Asexual sporulation of *T. atroviride*. (A) Quantitation of conidiation of the parent (P1), *Δlae1-1* and *OElae1* strains on PDA in light (white bars) and in darkness (full bars). Values are means of at least three independent biological experiments. Similar investigations with strain *Δlae1-2* yielded values within ± 8 % of those of *Δlae1-1.* All values are statistically different by the students t-test ($p < 0.05$).

Impairment of conidiation in the *T. atroviride Δlae1* strain cannot be rescued by volatile components from the parent strain

Conidiation in *Trichoderma* has been shown to be triggered by volatile compounds (VOC) from neighboring *Trichoderma* colonies (Nemcovic et al. 2008). We therefore surmised that the loss of conidiation in darkness could be due to a loss of the ability to form VOC. Consequently we tested whether VOC released by the parent strain of *T. atroviride* would rescue conidiation in the darkness in the *Δlae1* mutant. However, this hypothesis had to be rejected: using an upside-down sandwich of

two plates, in which the *Δlae1* mutant was growing in the plate on the top and the parent strain P1 on the bottom, the *Δlae1* strain maintained being unable to form conidia (shown for two mutants, *Δlae1-1* and *Δlae1-2* in **Figure 3**).

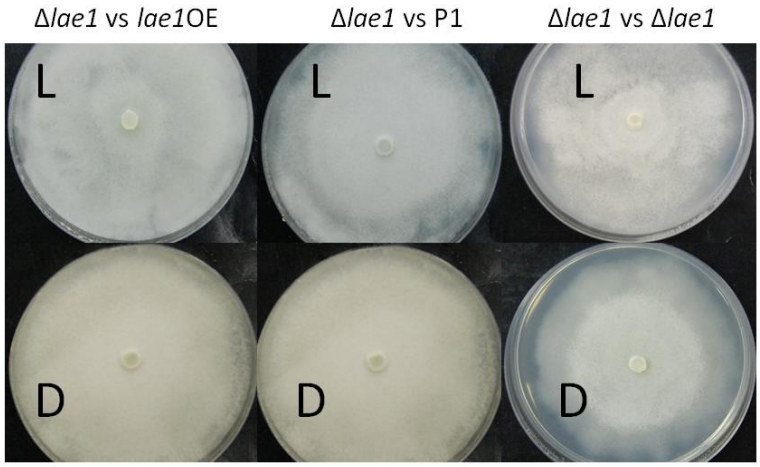


Fig. 3. Lack of induction of conidiation in *T. atroviride Δlae1* by volatiles from strains P1, *OElae1* and *Δlae1* (=control) in the presence of light (L) or in darkness (D).

There is no cross talk between LAE1 and the two blue light receptors BLR-1 and BLR-2

The significant effect of the *Δlae1* mutation on conidiation in response to light prompted us to investigate a possible cross-talk between LAE1 and the two blue light receptors BLR-1 and BLR-2, which form the top of the cascade that signals the presence of light to *T. atroviride* (Casas-Flores et al. 21). However, their transcripts were equally abundant in the parent, *OElae1* and *Δlae1* strains (**Figure 4A**) indicating that their expression is unaffected by *lae1* modulation. Also *lae1* was expressed at the same level in *Δblr1* and *Δblr2* mutants (**Figure 4B**). Hence, *lae1* and *blr1/blr2* do not influence the expression of each other.

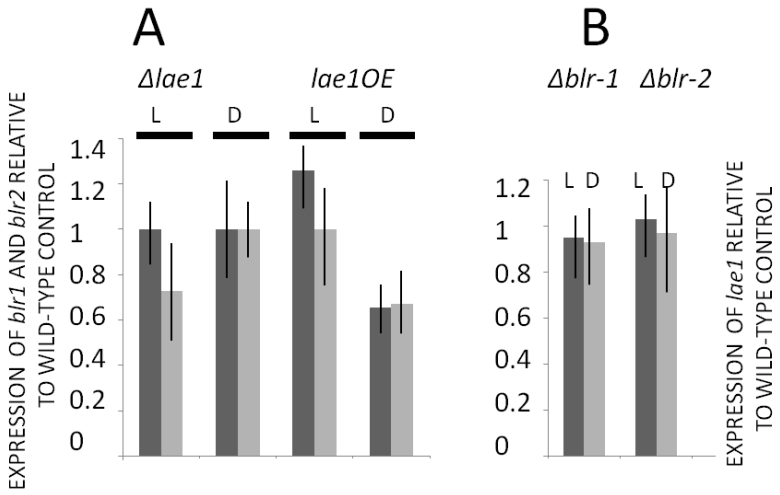


Fig. 4. (A) Expression of *blr1* (dark grey) and *blr2* (light grey) in *Δlae1-1* and *OElae1* in light (L) and darkness (D); (B) expression of *lae1* in Δ*blr1* and *Δblr2* in light (L) and darkness (D). Vertical bars indicate the standard deviation (N ≥ 3). Expression of *lae1, blr1* and *blr2* was normalized to the expression of *tef1*. Relative gene expression is calculated as the ratio of the normalized expression in the mutant in –fold of that of the parent strain P1. None of the difference was found to be statistically relevant by students t-test ($p > 0.15$).

LAE1 is also required triggering of conidiation by mechanical injury

Conidiation in *Trichoderma* can also be induced by mechanical injury via generation of radical oxygen species (ROS) (Casas-Flores et al. 2004, Steyaert et al. 2010, Carreras-Villaseñor et al. 2012, Hernández-Oñate et al. 2012). We have therefore investigated if LAE1 is also required for conidiation triggered by mechanical injury in darkness. As shown in **Figure 5,** mechanical injury resulted in conidiation only in the parent and *OElae1* strain but not in the *Δlae1* strain, and LAE1 therefore influences sporulation also when triggered by mechanical injury.

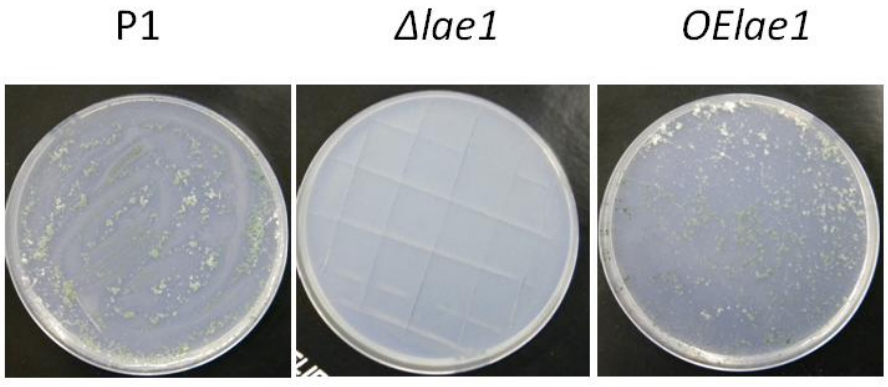


Fig. 5. Triggering of conidiation in the *T. atroviride* parent and *lae1* mutant strains by mechanical injury. The mycelium of the strains shown was cut with a scalpel and incubated under periodic illumination condition for 24 hrs. Single plates from several (N>4) experiments are shown.

LAE1 is required for oxidative stress tolerance in *T. atroviride*

Wu et al. (2012) recently showed LAE1 is necessary for the oxidative stress response in the plant pathogen *Cochliobolus heterotrophus*. To find out whether LAE1 is required for the response to oxidative stress in *T. atroviride*, we tested the effect of hydrogen peroxide on the *T. atroviride* parent, the *Δlae1* and the *OElae1* strain (**Figure 6**). The parent strain P1 proved to be resistant to hydrogen peroxide up to a concentration of at least 5 mM, and displayed about 60 % of its original growth rate at 20 mM. Similar data were obtained for the *OElae1* mutant. The *Δlae1* mutant, however, only showed 64 % of its growth rate at 5 mM hydrogen peroxide, and exhibited only 39 % of its original growth rate at 20 mM. Thus we conclude that LAE1 is partially involved in the defense against oxidative stress in *T. atroviride*.

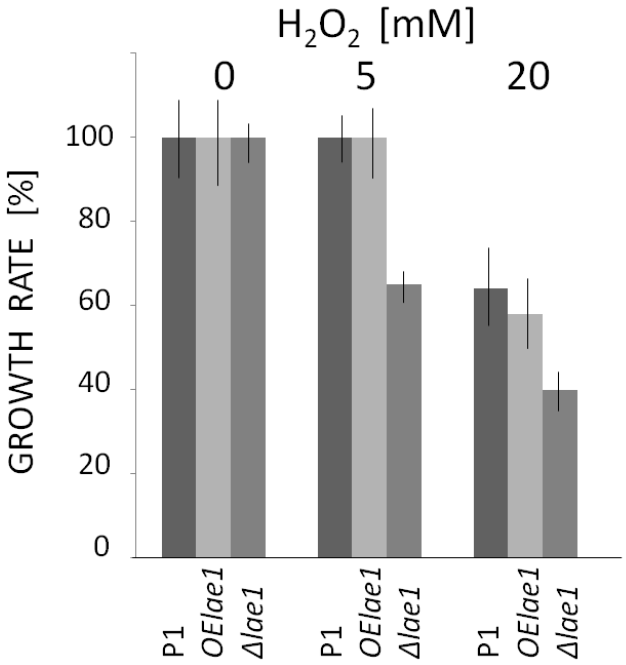


Fig. 6. Effect of hydrogen peroxide on growth of *T. atroviride* parent strain and *lae1* mutant strains. Growth on PDA was monitored in intervals of 6 – 12 hrs for a period of up to 100 hrs. Growth rates were calculated from the phase where the increase in colony diameter vs time was linear and were calculated as mm/h. In the figure, the growth rate of the strain in the absence of hydrogen peroxide was set to 100 %, and all other growth rates related to it. Data are means from at least 8 independent biological replicas.

LAE1 is essential for *T. atroviride* antagonism and defense against other fungi

To analyze whether LAE1 would be relevant for the mycoparasitic activities of *T. atroviride*, we confronted the parent strain P1, and the *Δlae1* and *OElae1* mutants on plates with three standard model fungi used for antagonism experiments (i.e. *Alternaria alternata, Rhizoctonia solani*, and

Botrytis cinerea). Their growth was monitored over the time in the presence and absence of *T. atroviride* and its *lae1* mutants (**Figure 7**). As can be seen, all three test fungi were initially able to grow in the presence of *T. atroviride* and its *lae1* mutants at the same rate as in their absence, but stopped their growth when getting close (1-2 mm) to *T. atroviride* (plates for the second mutant strains shown in **Figure 8**) This was about 50 h for all three fungi when confronted by strains P1 and OE*lae1*, whereas it occurred in the *Δlae1* mutant only after 65 hrs with *R. solani* and *B. cinerea* and 85 hrs with *A. alternata*. Correspondingly, the final colony diameter of these three fungi was higher when confronted with the *Δlae1* mutant than with P1 or OE*lae1*, which also corresponded with a smaller colony diameter of the *Δlae1* mutant strain. However, in addition to this slower growth of the *Δlae1* strain, visual examination of the plates (**Figure 8**) showed that it also failed to overgrow and feed on the tested plant pathogenic fungi, and in contrast its growth was suppressed by them. In confrontation with *R. solani*, *T. atroviride Δlae1* almost completely also lost its ability to conidiate. In contrast, the mycoparasitic vigor of the OE*lae1* strain was even increased, and we particularly noted an increased formation of coils around mycelia of *R. solani* (data not shown).

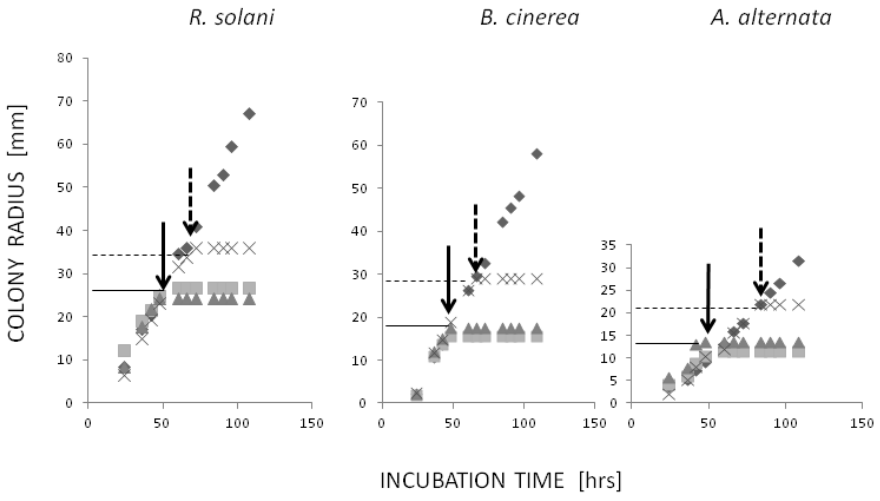


Fig. 7. Effect of modulation of *lae1* expression on the ability of *T. atroviride* to inhibit growth of *R. solani, B. cinerea* and *A. alternata*. A: (full ◊ indicate growth in the absence of *T. atroviride*; full Δ indicates growth in the presence of *T. atroviride* P1; full Δ shows growth in the presence of *T. atroviride* OE*lae1*; and x specifies growth in the presence of *T. atroviride Δlae1*. Full arrows define the time point where *T. atroviride* P1 and OE*lae1* stopped growth of the other fungi, whereas the dotted arrow specifies the time where *T. atroviride Δlae1* strain stopped fungal growth. The solid and dotted horizontal line show the respective biomass formed by the three test fungi at the time of inhibition. B: confrontation of *T. atroviride* strain Δ*lae1-2* with *R. solani, B. cinerea* and *A. alternata.*

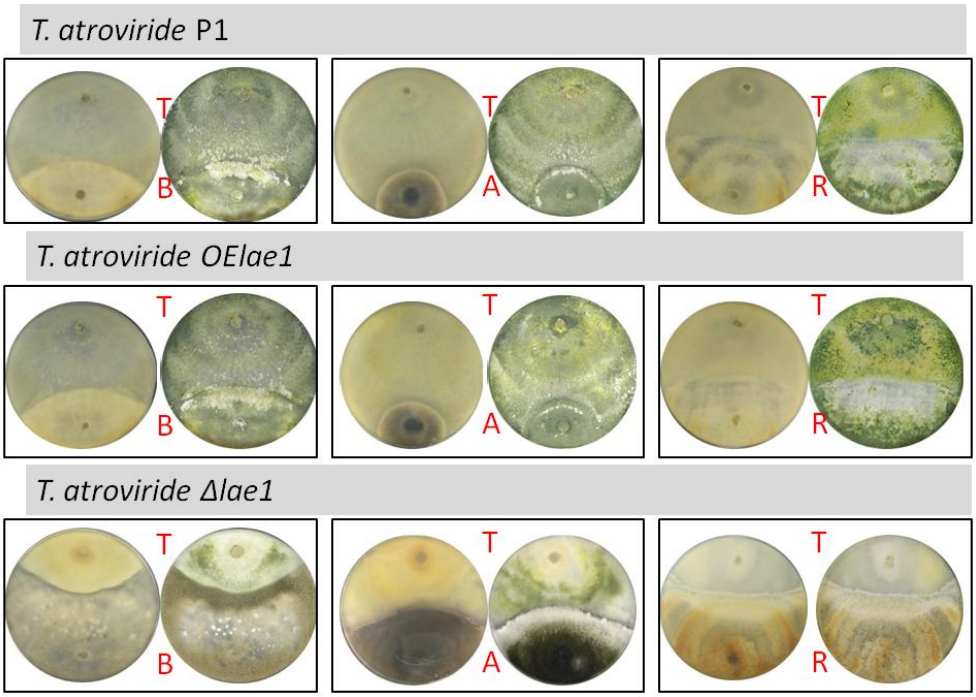


Fig. 8. Phenotype of confrontation of *T. atroviride* P1 and the *lae1* mutants OE*lae1* and *Δlae1* (all T) against *B. cinerea* (B), *A. alternata* (A) and *R. solani* (R) after termination of growth of the latter three fungi. Left plates are photographed from the backside, right plates are photographed from top.

LAE1 regulates the formation of extracellular antifungal components

The dependence of mycoparasitism and antagonism on LAE1 prompted us to test whether this could be due to an involvement of LAE1 in the formation of water soluble extracellular compounds (WSC) that aid in the inhibition of growth of the plant pathogenic fungi. To this end, we grew *T. atroviride* P1, and its *Δlae1* and OElae1 mutants on plates covered by cellophane. After *T. atroviride* had covered most of the plates, the fungal mycelium and the cellophane were removed, and *A. alternata, R. solani* and *B. cinerea* inoculated into the middle of these plates. As shown in **Figure 9**, the three fungi failed to grow on the plates on which the parent strain or the OE*lae1* mutant had been pregrown, whereas they were still able to grow on plates on which the *Δlae1* had been grown. Yet the latter growth was nevertheless clearly slower then on plates not precolonized by *Trichoderma*. Consequently we conclude that LAE1 contributes to but is not essential for the formation of extracellular antifungal compounds by *T. atroviride*.

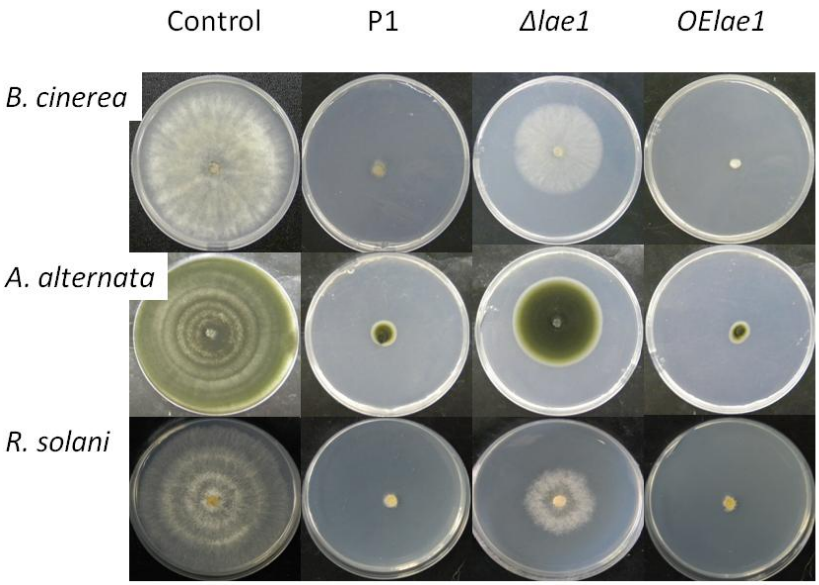
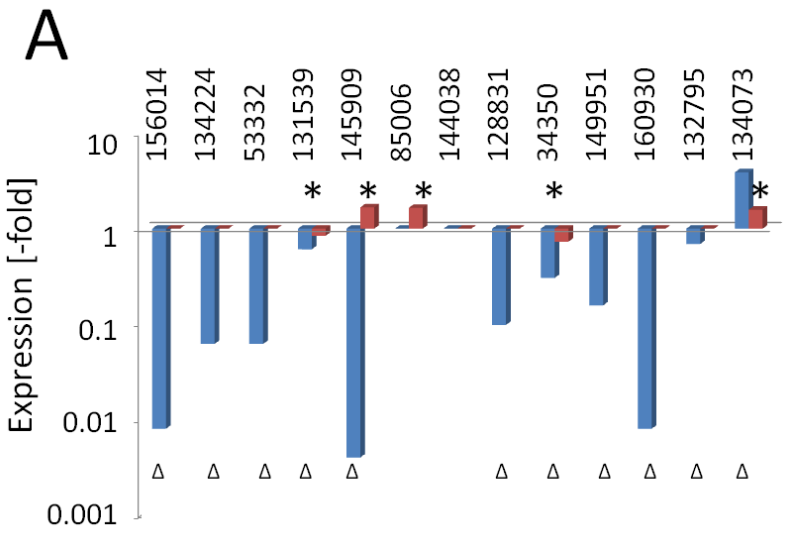


Fig. 9. Test for production of WSC: *T. atroviride* parent strain, and the *Δlae1-1* and OE*lae1* mutants were grown on PDA agar covered by cellophane, and then removed and *Alternaria alternata* (*Aa*), *Rhizoctonia solani* (*Rs*) and *Sclerotinia sclerotiorum* (*Ss*) placed on these plates. The plates were photographed 1-2 mm before that the control has reached the edge of the plate.

Loss of function of *lae1* decreases the expression of mycoparasitism-associated genes

In order to learn whether loss of function of LAE1 would be due to a decreased expression of genes known to be associated with mycoparasitism, we have investigated the expression of 13 genes that were recently shown to be strongly upregulated during interaction of *T. atroviride* with *R. solani* (Atanasova et al. 2012). These were two GH16 ß-1,3/1,4-glucanases, two aspartyl proteases, two subtilisin proteases, two polyketide synthases, two C-type lectins, one cyanovirin-type lectin and two small cysteine-rich secreted proteins. **Figure 10A** shows that indeed 8 of these 13 genes were significantly underexpressed in the *Δlae1* mutant. Interestingly, the expression of none of these genes was enhanced in the OE*lae1* strain, implying that the superior mycoparasitic activity of this mutant (*vide supra*) cannot be due to the increased expression of any of these genes. Nevertheless, the data demonstrate that in *T. atroviride* LAE1 is necessary for the expression of some of the genes encoding extracellular hydrolases, secondary metabolites and proteins that putatively interact with other organisms.



Fig, 10. Modulation of expression of genes putatively involved in mycoparasitism (A). Ratios of expression between the parent strain and either the *Δlae1-1* (blue bars) or the OE*lae1* strain (red bars) are shown. The * and Δ symbols indicate $p < 0.05$. Genes are given by their Triat2 number: 34350, GH16 ß-glycosidase; 144038, aspartyl protease; 160930, aspartyl protease; 128831, C-type lectin; 132795, C-type lectin; 134073, cyanovirin-N; 156014, GH16 ß-1,3/ß-1,4-glucanase; 85006, polyketide synthase (PKS); 53332, small cystein-rich secreted protein (SSCP); 131539, SSCP; 145909, subtilisin-like protease; 149951, subtilisin-like protease. Data are plotted relative to wild-type (P1) control.

LAE1 affects γ-pentyl-pyrone formation by *T. atroviride*

One of the known antifungal metabolites produced by *T. atroviride* is 6PP (Reino et al. 2008, Claydon et al. 1987), which exhibits an intensive coconut smell. During the antagonism experiments, we observed this aroma in plates of the parent strain and even more in plates of the *OElae1* strain of *T. atroviride*, whereas it was absent from those of the two *Δlae1* strains and only appeared faintly when this culture initiated its sporulation in the presence of light. To experimentally test whether LAE1 indeed regulates 6PP formation, we examined the expression of the lipoxygenase gene (Triat2:33350) that is putatively involved in 6PP formation (Kubicek et al. 2011), and which is strongly upregulated during mycoparasitism (Atanasova et al. 2012). In fact, the lipoxygenase transcript was strongly down regulated in *Δlae1*, both in ambient light and in dark. It was also upregulated in OE*lae1* in the dark but not in light (**Figure 10B**).

To test whether reduced 6PP formation would contribute to the reduced antagonistic activity in the *T. atroviride Δlae1* strain, we tested the effect of VOC from the parent strain, the OE*lae1* and the *Δlae1* strain on growth of the test fungi. The data show that VOC indeed reduce the growth of *R.*

solani and also of *A. alternata* (data not shown), but only partially, and that this effect is much weaker in the *Δlae1* strain (**Figure 10C**).

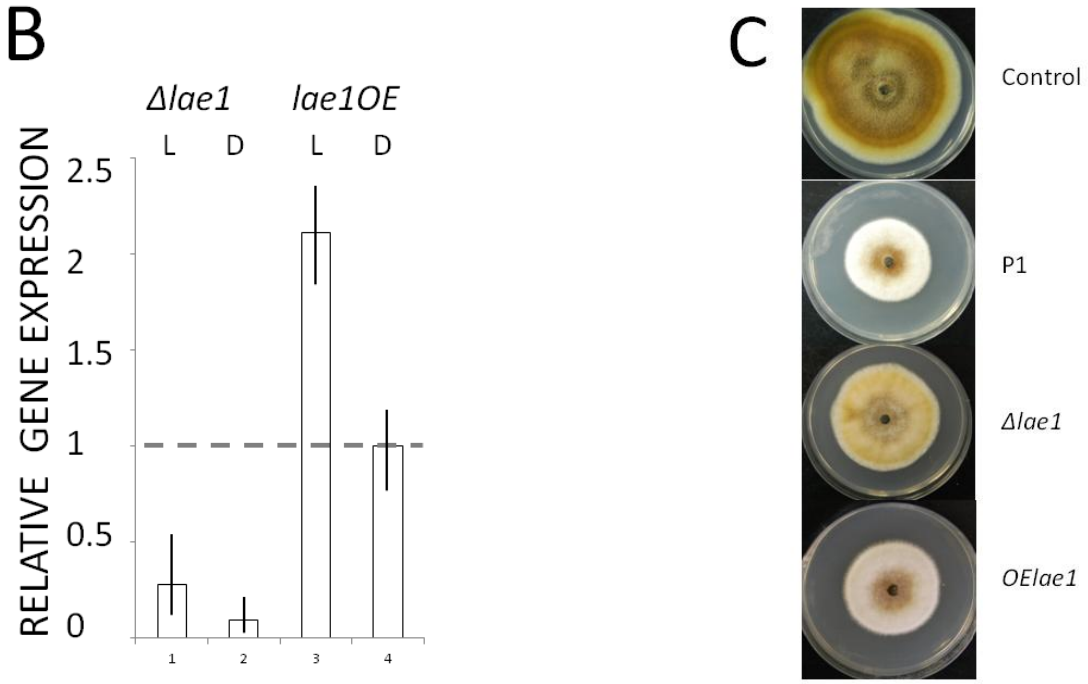


Fig. 10. (B) Expression of the lipoxygenase gene in the *Δlae1* and OE*lae1* strains in light (L) and darkness (D). (C) Growth of *R. solani* in the presence of VOC from the *T. atroviride* parent strain and the *lae1* mutants. Only *A. alternata* is shown but essentially similar results were also obtained with *R. solani* and *B. cinerea.* Single plates from several (N>4) experiments are shown.

Discussion

The putative protein methyltransferase LaeA is still an enigmatic protein: it has originally been identified as a regulator of secondary metabolite (aflatoxin) biosynthesis (Bok et al. 2004), but subsequently was found to have – among other traits – also key functions in development: as an example, deletion of the *laeA* gene in *A. fumigatus* and *A. flavus* reduces conidiation (Bok et al. 2005, Chang et al. 2012). In *P. chrysogenum* and in *A. nidulans*, this impairment of asexual sporulation is seen both in light as well as in darkness (Hoff et al. 2010, Karimi-Aghcheh et al. 2013), whereas in *C. heterostrophus*, the *Chlae1* mutant was relieved from repression of conidiation in the dark and produced numbers of conidia similar to wild-type in light (Wu et al. 2012). In *T. reesei*, whose sporulation is not enhanced by light (Seiboth Karimi et al. 2012, Druzhinina et al. 2010), formation of

conidia is reduced to almost zero in both light and darkness. In the present study, we found that the *T. atroviride Δlae1* mutant fail to conidiate in the dark on all carbon sources , while conidiation – which is stimulated by light (Steyaert et al. 2010, Carreras-Villaseñor et al. 2012, Friedl et al. 2008) - was only reduced by 50 % in light. Thus, while LaeA/LAE1 all affect asexual sporulation, the necessity for a functional LAE1 in *T. atroviride* prevails predominantly in darkness and appears to be partially counteracted by light, which is a unique case in the fungi studied so far.

One special trait of *Trichoderma* conidiation is that it can be induced by mechanical injury of the mycelium (Casas-Flores et al. 2004, Steyaert et al. 2010, Carreras-Villaseñor et al. 2012, Hernández-Oñate 2012). The present results showed that injury-triggered sporulation of *T. atroviride* in the dark was also dependent on LAE1 function, as no conidia were formed in its absence. Mechanical injury has been shown to be triggered by an oxidative stress response caused by NADPH oxidase-dependent production of radical oxygen species (ROS), for which the proteins NOX1 and NOXR are essential (Hernández-Oñate et al. 2012). We have not tested whether the expression of *nox1* and *noxR* is affected by LAE1 loss-of-function in *T. atroviride* (the *T. reesei* orthologues are not; cf. [Karimi-Aghcheh et al. 2013]) but tested whether – as in *C. heterostrophus* (Wu et al. 2012) – the *Δlae1* strain is affected in its sensitivity to oxidative stress provoked by hydrogen peroxide. While we found that this indeed the case, the reduction of resistance against hydrogen peroxide was not complete and required much higher concentrations than in *C. heterostrophus* (Wu et al. 2012). We therefore conclude that – even in the absence of *lae1* function - *T. atroviride* can still respond to oxidative stress. The complete loss of conidiation upon mechanical injury in the *Δlae1* strain must therefore be due to a requirement for LAE1 by other components needed for this process.

One of them could be VOC formation: sporulation by *T. atroviride* has been shown to be triggered by VOC from other *Trichoderma* colonies (Nemcovic et al. 2008), and Roze et al. (2010) showed that in *A. parasiticus*, the Velvet A protein VeA is required for production of VOC that mediates asexual conidiation and sclerotia formation. Because of the known interaction of VeA with LaeA (Bayram et

al. 2012, Bayram et al. 2008), which has also been shown in *T. reesei* (Karimi-Aghcheh et al. 2013), we tested whether LAE1 would be involved in the stimulation of *T. atroviride* sporulation by VOC. However, VOC from the parent strain were unable to overcome the reduced conidiation in *Δlae1*. Thus, either the effect of VeA on induction of sporulation is independent of LaeA (e.g. in *P. chrysogenum*, the VeA and LaeA orthologues PcVelA and PcLaeA have different and independent roles in asexual development [Hoff et al. 2010]), or this process is differentially regulated in *A. parasiticus* and *T. atroviride*.

The most striking and not yet reported phenotype of loss-of-function of LaeA is that the *T. atroviride Δlae1* strain had completely lost its mycoparasitic ability, and also the ability to defend itself against other fungi. This is similar to data that have been reported for *Δvel1* strains of *T. virens*, suggesting that mycoparasitism is indeed controlled by the VEL1/LAE1/VEL2 (VeA/LaeA/VelB) complex (Bayram et al. 2012, Bayram et al. 2008). We should like to stress that *lae1* and *vel1* are so far the only genes that have been identified as global regulators of *Trichoderma* antagonism, and whose loss-of-function is not compromised by severe growth defects: deletion or silencing of other genes, such as those encoding G-proteins or their receptors, while also leading to impaired mycoparasitic activity, also caused significant reductions in the growth rate of *Trichoderma* (Omann et al. 2010) and it is therefore difficult to assess whether their effect on mycoparasitism is direct or indirect. Although the *T. atroviride Δlae1* strains showed some reduction of growth on some carbon sources, the effects did not exceed ± 30 % of that of the parent strain under conditions of antagonism with the test fungi, and particularly the hyphal morphology was not significantly altered (data not shown). We therefore do not believe that the loss of antagonistic abilities could be solely due to this fact.

Interestingly, qPCR analyses showed that this loss of mycoparasitic ability correlated with a loss of expression of genes encoding cell wall hydrolases (GH16 glucanases), secondary metabolites (PKS), and proteins supposed to mediate hyphal contact to the host (lectins, SSCPRs). This would be in excellent agreement the current view of mechanism of *Trichoderma* mycoparasitism (Druzhinina et

al. 2011). However, none of these genes displayed enhanced expression in the OE*lae1* strain, and the superior mycoparasitic activity in OE*lae1* strains thus remains unexplained. We have recently observed that overexpression of *lae1* even converts other *Trichoderma* spp. that exhibit only weak antagonistic activities, into vigorous mycoparasites (R. Karimi-Aghcheh, M. Marzouk and I.S. Druzhinina, unpublished data). LAE1 therefore must act at a target that is central to the mycoparasitic response that still awaits identification.

Work on LaeA and its orthologues in several Aspergilli, but also in *P. chrysogenum, Fusarium fujikuroi, F. verticillioides* and *C. heterostrophus* has consistently proven that it regulates secondary metabolism (Hoff et al. 2010, Wiemann et al. 2010, Wu et al. 2012, Bok et al. 2005, Sugui et al. 2007, Friedl et al. 2008). However, only a few genes of secondary metabolism were affected by a loss of function of *lae1* in *T. reesei* (Karimi-Aghcheh et al. 2013), suggesting that the function of LAE1 may have diverged in this genus. The present investigation with *T. atroviride* supports this view: while some of the secondary metabolism genes that have recently been shown to be upregulated during antagonism of *T. atroviride* against *R. solani* (Atanasova et al. 2012) were significantly underexpressed in the *Δlae1* mutant, there was a less strong effect of LAE1 on the formation of inhibitory WSC and VOC, thus implying that the *Δlae1* strain still can form WSC and VOC. Whether this is due to a decreased expression of several genes or the complete blockage of expression of some of them remains to be determined. One must also bear in mind that the tests for WSC and VOC formation depends on the prior cultivation of *T. atroviride* in the absence of its prey, and it is possible that stronger effects may become apparent when the formation of these components is investigated under confrontation conditions. Yet it is clear from these studies that the lack of mycoparasitic activity in the *Δlae1* strain cannot be explained by the observed changes in the expression of its secondary metabolism genes.

The present findings that LAE1 regulates the antagonistic and defensive reaction of the mycoparasite *T. atroviride*, is a further example of involvement of this protein in a specific response of a fungus to

the environment. A similar conclusion has also been drawn by Sarikaya Bayram et al. (2010), i.e. that LaeA is involved in the protective as well as the nutritional function for preparing the next generation for future life. Such a role would be in excellent agreement with an epigenetic function of LaeA/LAE1 (Schrey and Richards 2012), which however so far is only a speculation.

Materials and Methods

Fungal strain and culture conditions

Trichoderma atroviride P1 (ATCC 74058; [Kullnig et al. 2001]) was used throughout this work. For selected experiments, *T. atroviride* IMI 206040, and its *blr1* and *blr2* deletion mutants (Casas-Flores et al. 2004) were also used. It was grown on PDA (Difco^{TM} potato-dextrose-agar) plates at 25°C. *Rhizoctonia solani* C.P.K. 3753, *Botrytis cinerea* C.P.K. 4679 and *Alternaria alternata* C.P.K 3737 were grown on 2% (w/v) potato dextrose agar (PDA) under 12 h cycles of light and darkness at 25 °C.

Escherichia coli JM109 (Promega, Madison, Wisconsin) was used for plasmid construction and amplification.

Manipulation of *lae1* gene expression in *T. atroviride*

To obtain mutants not expressing *lae1*, the 1.2 kb *lae1* coding region was replaced by the hygromycin B phosphotransferase (*hph*) gene from *E. coli* under *Trichoderma* 5' and 3' regularory signals (Mach et al. 1994). To this end, 1.3 and 1.2 kb of the up- and downstream non-coding region of *lae1* were amplified using the primer pairs Patro_FW_ConMeth_ApaI (5'-TGGGCCCCATCATATCTGCTACTTGGCTC-3') / Patro_Rev_ConMeth_XhoI (5'-TCTCGAGCGAGTATGGCGAGTCCTATAG-3'), and Tatro_FW_ConMeth_XhoI (5'-TCTCGAGGACCTAACCCGCATTACTTTG-3')/ Tatro_Rev_ConMeth_SmaI (5'-TCCCGGGCATCAAGAGCGTAGCACTG-3') respectively. The two resulting PCR fragments were digested

with *Apa*I/*Xho*I (upstream region) and *Sma*I/*Xho*I (downstream region), dephosphorylated and ligated into pBluescript SK(+) (Stratagene, La Jolla, California), previously cut with *Apa*I/*Sma*I, followed by the insertion of the 2.4 kb *Xho*I/*Sal*I fragment of *hph* casette into the *Xho*I site resulting in pRKA_D 42103hph.

Vector pRKA_OE41617hph, which bears the *T. reesei lae1* gene under the constitutive expression signals of *tef1* (Akel et al. 2009) was used to generate *lae1* overexpressing strains of *T. atroviride*.

Transformation of *Trichoderma*

Transformation has been carried out as described by Guangtao et al (2009). The strains were purified twice to obtain mitotic stability, and integration of the expression cassettes was verified by PCR analysis (**Figure 1**).

Assay for growth and conidiation

Cultures were grown on PDA at 25 °C in a Sanyo incubator containing a Philips-master light source (TLD-15 W/840), either with illumination (12 hour cycles of light and dark; 1100 [± 30] lux, 30 cm distance) or in full darkness (dark conditions), as specified. To this end, each plate was inoculated with a mycelial plug (5 mm diameter) taken from the edge of a 3-day-old non-sporulating plate. Three replica were done for each treatment. Conidia were harvested by gently rubbing them off in an equal volume of physiologically salt (0.1%, w/v, Tween and 0.8% w/v NaCl), filtering through glass wool, and centrifugation (5000 x g, 10 min). The conidia were then suspended in 2.5 g/l phytagel (Phytagel^{TM,} SIGMA, Steinheim, Germany), mixed and their transmission measured at 590 nm in a Biolog standard turbidimeter. The number of conidia was calculated using a calibration curve with *T. reesei* conidia.

Phenotype microarrays

Growth of *T. atroviride* P1 and the *lae1* mutant strains derived from it on 95 carbon sources and water was investigated using Biolog® phenotype microarrays as described by Friedl et al (2008). One-way or main-effect analyses of variance (ANOVAs) were used to compare the growth of selected strains on individual carbon sources. Tukey's honest significant difference (Tukey HSD) test as implemented in STATISTICA 6.1 was used for post hoc comparisons to detect the contribution of each variable to the main effect of the F test resulting from the ANOVA. Only p-values < 0.05 were considered as significant.

Assays for fungal antagonism

The ability of *T. atroviride* P1 and the respective recombinant mutants to antagonize and eventually parasitize other fungi was tested in dual confrontation assays on PDA plates as described earlier (Atanasova et al. 2012). To study the ability to coil around hyphae of other fungi, 2% sucrose nutrient agar (SNA) (1.0 g/l KH_2PO_4, 1.0 g/l KNO_3, 0.5 g/l $MgSO_4.7H_2O$, 0.5 g/l KCl, 0.2 g/l glucose, 0.2 g/l sucrose) was used, and the interaction monitored under the light microscope at the zone of interaction after 10, 14 and 21 days.

To test for a possible involvement of water soluble secreted compounds in antagonism, the *T. atroviride* strains were grown on PDA plates covered by cellophane. After the strains had covered approximately two thirds of the plates, the cellophane with *T. atroviride* was removed, and *A. alternata, R. solani* and *B. cinerea* were inoculated into the middle of these plates, and incubation continued until the respective controls reached 2 mm distance from the edge of the plates.. The respective colony radius was monitored and compared to a control where the same fungus was pregrown.

Triggering conidiation by mechanical injury

To induce conidiation by mechanical injury (Casas-Flores et al. 2004), *T. atroviride* and its mutants were grown at 25 °C on plates containing either minimal medium, Vogel's medium or SNA in darkness. After the mycelium had spread over the whole plate, it was cut with a sterile cold scalpel and incubated in darkness for additional 24 – 48 hrs.

Testing the effect of volatile compounds (VOC)

T. atroviride P1 and its *Δlae1* mutants were grown on PDA at 25 °C for 72h in periodic light and in darkness. Then the dishes were opened under sterile conditions and two dishes each arranged as an upside-down sandwich, in which the *Δlae1* mutant plate was on the top. The sandwich was sealed with Parafilm tape and the incubation continued at 25 °C for 8 days. Control plates (i.e. both plates contained the same strain) were always included.

The same test was applied when the effect of VOC on other fungi was tested. *A. alternata, R. solani* and *B. cinerea* were grown in the top plate.

Hydrogen peroxide sensitivity

To test for sensitivity of the *Trichoderma* strains against hydrogen peroxide, they were grown on PDA plates supplemented with 0, 0.5, 1, 5 and 20 mM of H_2O_2, and incubated until the time that the fungi reached the edge of the plates. Changes in radial growth were measured 3 times per day.

Assay for cellulase formation

The *Trichoderma* strains were grown in Mandels-Andreotti medium (Mandels & Andreotti 1978) containing 1% (w/v) carboxymethyl cellulose (CMC) as carbon source on 2 % (w/v) agar plates. The

inoculated plates were then incubated at 25°C for 3 days. Thereafter, they were incubated at 50 °C for 18 hrs. The hydrolyzed cellulose was detected by staining with a 0.1% (w/v) Congo-Red solution, followed by subsequent washing with 1M NaCl. The hydrolysis of cellulose becomes visible by the bright halos around the fungal colony, and expressed as ratio of the diameter of the halo to that of the fungal colony. A ratio of 1 indicates absence of cellulase formation.

Quantitative PCR

Following RNA isolation (using the RNeasy plant kit, Promega) 5 µg of the total RNA was treated with DNAse (DNase I, RNase free; Fermentas) and reverse transcribed (RevertAid™ First Strand cDNA Kit, Fermentas) using a 1:1 mixture of oligo-dT and random hexamer primers. All quantitative RT-PCR experiments were performed on a Bio-Rad (Hercules, CA) iCycler IQ. For the reaction the IQ SYBR Green Supermix (Bio-Rad, Hercules, CA) was prepared for 25 ml assays with standard $MgCl_2$ concentration (3 mM) and a final primer concentration of 100 nM each. All assays were carried out in 96-well plates. Determination of the PCR efficiency was performed using triplicate reactions from a dilution series of cDNA (1; 0.1; 0.01; 0.001). Amplification efficiency was then calculated from the given slopes in the IQ5 Optical system Software v2.0. Primers, amplification efficiency and T_m values are given in Table A1 (Appendix 3)**.** Expression of the reference gene *tef1* was measured with both protocols for reference calculation. Expression ratios were calculated using REST© Software (Pfaffl et al. 2002). All samples were analyzed in at least two independent experiments with three replicates in each run.

Acknowledgements

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Chapter 6

The VELVET A Orthologue VEL1 of *Trichoderma reesei* Regulates Development and Acts as an Auxiliary Component of Cellulase Gene Expression

The results presented in this chapter have been submitted to Appl Microbiol Biotechnol (AMB)
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VEL1 controls sexual and asexual development independent of the presence of light and acts as an auxiliary component of cellulase gene expression

Abstract

We have recently shown that expression of cellulase and hemicellulase encoding genes in *Trichoderma reesei* is obligatorily dependent on the function of the protein methyltransferase LAE1. Its *Aspergillus nidulans* orthologue LaeA is a part of the VELVET protein complex consisting of LaeA, VeA and VelB that regulates secondary metabolism and sexual reproduction. Here we have investigated a possible role of VEL1, the *T. reesei* orthologue of *A. nidulans* VeA, in *T. reesei* development and cellulase gene expression. Expression of *T. reesei vel1* gene occurs at a low level, which is regulated by the carbon source, and in most cases higher during cultivation in the dark. Deletion of the *T. reesei vel1* locus causes a complete loss of conidiation in light and darkness and loss of formation of perithecia during mating in the presence of light. Overexpression of *vel1* under the constitutive expression signals of *tef1* did not significantly enhance conidiation in light or darkness, and led to infrequent formation of infertile perithecia in the dark. Deletion of *vel1* did not affect cellulase gene expression, but *vel1* overexpression strongly enhanced it. Consistent findings were also obtained for the formation of xylanase and ß-xylosidase enzyme activities. The stimulation of cellulase gene expression by overexpressing *vel1* was dependent on a functional *lae1* allele. Our data show that VEL1 controls sexual and asexual development independent of the presence of light. In addition, while *vel1* overexpression stimulates cellulase gene expression, its knock out does not impair it and its action must therefore be different from that of LAE1.

Introduction

Cellulose and hemicelluloses form the major amount of plant biomass and thus represent the largest reservoir of renewable carbon sources on Earth, which could potentially replace fuels and refinery products derived from fossil carbon components (Kubicek 2012a). To this end, efficient hydrolysis of the plant cell wall polymers to soluble oligo- and monomers is essential. The Sordariomycete *Trichoderma reesei* (teleomorph *Hypocrea jecorina*) is most widely used for the industrial production

of cellulolytic and hemicellulolytic enzymes and has become a basis for the modern paradigm applied to these enzymes (Kubicek 2012b). The *T. reesei* genome encodes 2 cellobiohydrolases, six endo-ß-1,4-glucanases and 16 hemicellulases (Martinez et al. 2008). Most of these genes are regulated in a consistent manner, and are synthesized only in the presence of an inducer, which can be either cellulose itself, disaccharides generated by its degradation (such as sophorose) or the galactosyl-β-1,4-glucoside lactose (Foreman et al. 2003, Seiboth et al. 2012), and which is dependent on the function of XYR1 (xylanase regulator 1), the main activator of cellulase and hemicellulase gene expression (Stricker et al. 2008, Seiboth et al. 2012). We have recently shown that the expression of genes for lignocellulose degradation in *T. reesei* is further – and independent of XYR1 - obligatorily dependent of the function of the protein methyltransferase LAE1, the orthologue of the *A. nidulans* regulator of secondary metabolism and development LaeA (Seiboth Karimi et al. 2012). LaeA is believed to exert an epigenetic control function by counteractinfg H3K9 methylation in the sterigmatocystin gene cluster (Bok et al. 2006, Reyes-Dominguez et al. 2010). Conversely, no LAE1-dependent changes in H3K9 methylation were observed in *T. reesei* (Seiboth Karimi et al. 2012, Karimi-Aghcheh et al. 2013) thus raising the question as to the mechanism by which LAE1 stimulates cellulase gene expression.

LaeA is a part of the trimeric VELVET protein complex, that consists of LaeA, VeA and VelB and that regulates secondary metabolism and sexual reproduction in *A. nidulans* (Bayram et al. 2008, Bayram and Braus 2012). In this fungus, the founding member of the complex, VeA, regulates several cellular processes, such as asexual and sexual development and biosynthesis of secondary metabolites (Calvo 2008, Bayram and Braus 2012). Most studies on *veA* have been carried out in *Aspergillus* spp., where this gene has been described to control photodependent development (Champe et al. 1981, Yager 1992, Kim et al. 2002, Duran et al. 2008, Calvo 2008, Purschwitz et al. 2008). Recently, the VeA orthologue VEL1 of *Trichderma virens* has been shown to regulate sporulation, chlamydospore

formation, secondary metabolite synthesis and mycoparasitism in this fungus (Mukherjee and Kenerley 2010).

Our finding that LAE1 is essential for cellulase gene expression (Seiboth Karimi et al. 2012) raised the question whether this action requires a functional VELVET complex. To test this, we have cloned and functionally characterized the VeA orthologue of *T. reesei*, VEL1. We will show that *T. reesei vel1* – in contrast to *lae1* – is not essential for cellulase gene expression, but its overexpression in the presence of functional *lae1* gene further stimulates cellulase formation. In addition, we will show that *vel1* is essential for asexual and sexual development of *T. reesei*.

Results

The VEL1 orthologue of *T. reesei*

The genome of *T. reesei* contains a single copy of the *vel1* gene (Trire2:122284; Gene Bank accession number of the respective protein VEL1 EGR48103.1). The ORF of *vel1* consists of 1,801 bp, is interrupted by a single 79 bp intron and encodes a 574 amino acid protein. Inspection of the genome sequences of the improved cellulase producer strains QM 9414, NG14 and RUT C-30 (Le Crom et al. 2009, Vitikainen et al. 2010) showed that they contain gene copies with identical nucleotide sequences, proving that *vel1* has not been altered by mutagenesis towards improved cellulase formation.

Blastp analysis revealed that VEL1 is highly similar to the VeA orthologues in *T. virens* and *T. atroviride* (80 and 78 % similarity over the entire amino acid sequence respectively). Highest similarity outside of the genus was observed with *Nectria haematococca* (64 %), whereas it was only 36 and 38% with *A. nidulans* and *A. fumigatus*, respectively. In accordance with studies in *A. nidulans* and *Neurospora crassa* (Kim et al. 2002, Bayram et al. 2008, Stinnett et al. 2007), WoLF PSORT identified the protein to be nuclear, the responsible motif being located at the N-terminus, and NES

identified a putative nuclear export signal in the C-terminal quarter of the protein sequence. Like *A. nidulans* VeA, the *T. reesei* VEL1 protein also contains a proline-rich potential PEST region (HAPPPLPPPPPSSYDAPPPAAR; PEST score 9.97), but in contrast to *A. nidulans*, where it is located at the C-terminal end of the protein (Kim et al. 2002), *T. reesei* VEL1 displays it in the middle of the protein immediately after the conserved N-terminal half (aa 290-311).

Expression of *T. reesei vel1* is carbon source dependent

We have examined the expression of *vel1* during hyphal growth on plates and subsequent sporulation on three different carbon sources and in light and darkness. During early growth and in the presence of light, *vel1* appears to be constitutively expressed on a rather low level (**Figure 1**). However, its expression changed in a carbon source dependent manner during longer cultivation and at the time of conidiation (35 hrs for all three carbon sources in **Figure 1**): whereas it increased on glucose throughout cultivation, and even stronger on lactose before conidiation, it remained at the same level on glycerol. Different findings were observed with cultures growing in complete darkness: with the exception of the first time point, growth on glucose and glycerol in the dark resulted in significantly higher levels of the *vel1* transcript. In contrast, *vel1* expression in the dark was highest on lactose during vegetative growth, but decreased at the onset of sporulation (**Figure 1**).

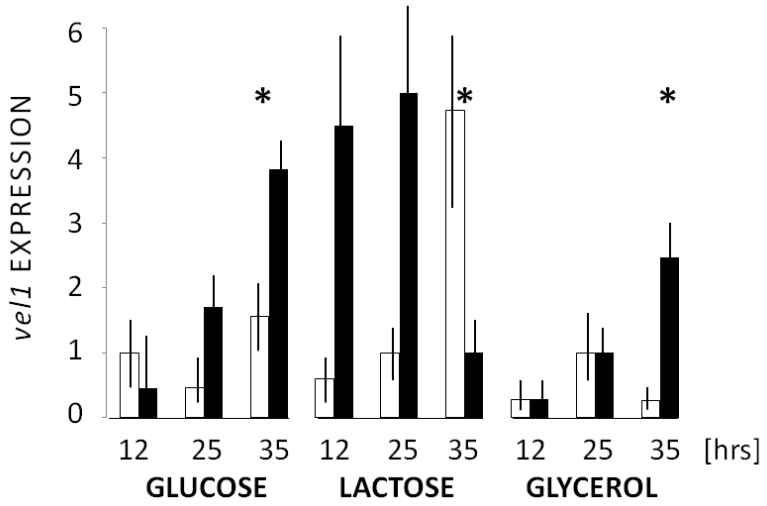

Fig. 1. Transcription of *vel1* during growth on glucose, glycerol and lactose in the presence of ambient light (white bars) and darkness (full bars). Gene expression is given in arbitrary units, which were calculated by normalizing the *vel1/tef1* ratio to that on glucose (12 h, ambient light). Data are means of at least 3 biological replicas, and only results with $p < 0.05$ are shown. The asterisk indicates the time point where the cultures started to sporulate (this time point was the same in light and darkness).

VEL1 is essential for sexual and asexual development in *T. reesei*

To investigate the impact of *vel1* on the development of *T. reesei*, *vel1* null mutants (Δ*vel1*) were generated by replacing the *vel1* coding region with the *E. coli* hygromycin B phosphotransferase gene *hph* in the *T. reesei* QM 9414. In addition, we generated overexpressing (*vel1*OE) mutants by fusing the *vel1* ORF downstream of the strongly expressing *tef1* (elongation factor 1α gene) promoter. Respective mutant strains were purified and verified by PCR (**Figure 2**).

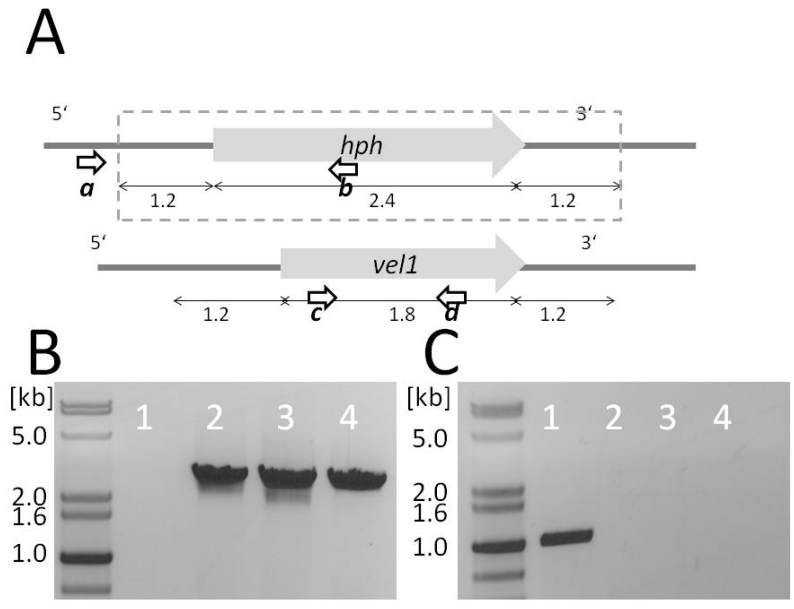

Fig. 2. PCR verification of *vel1* knock out in *T. reesei*: (A) structure of the disrupted (top) and native *vel1* locus (below). Numbers indicate the size (in kb) of the respective areas. The dotted line defines the gene construct present in the deletion cassette. The arrows ***a*** **–** ***d*** specify the primers used for amplification the homologous integrated knock-out construct (a and b; result shown in B), and of the native *vel1* gene (c and d; result shown in C), respectively. ***a***, pVel1; ***b***, hph_int; ***c***, Vel_int1; ***d***, Vel_int2 (for sequences see Table A1). Tracks: 1, parent strain QM9414; 2, *Δvel1* strain RKA14, 3, *Δvel1* strain RKA17, *Δvel1* strain RKA18.

Despite several trials, we were unable to obtain retransformants and therefore used three Δ*vel1* strains for the analysis. qPCR confirmed that they only had a single copy of the deletion construct integrated into the genome (data not shown). The copy number of the *vel1*OE strains was determined by Southern analysis (**Figure 3**), and two of them used for further investigations.

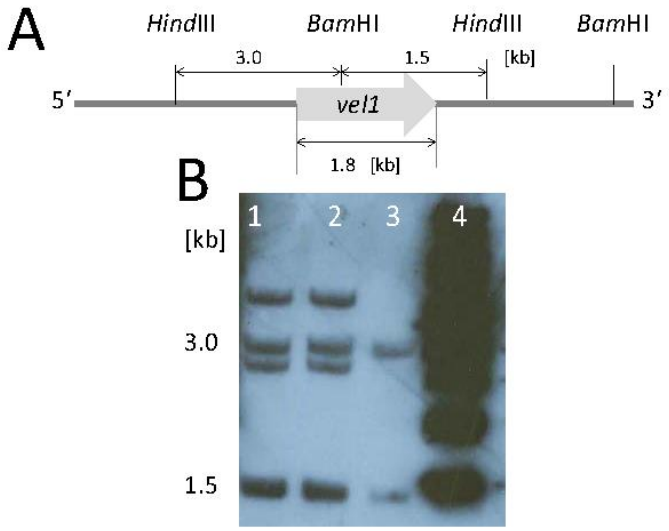

Fig. 3. Southern analysis of the *vel1*OE strains. A, scheme of the wild-type *vel1* locus. DNA was cleaved by *Hind*III and *BamH*I, and hybridization was done by a full-length 1.8 probe of *vel1*. B, resulting autoradiograph: Tracks: 1, RKA12; 2, RKA13; 3, parent strain QM9414; 4, size marker ladder.

The phenotype of the mutants on plates was different to that of the Δ*lae1* strains (Seiboth Karimi et al. 2012), as the Δ*vel1* strains were almost completely impaired in conidiation, both in light as well as in darkness. Yet they still produced the yellow pigment that is characteristic for *T. reesei* and not formed by Δ*lae1* strains. In addition, the *vel1*OE strains were not hypersporulating but formed conidia at a similar intensity as the parent strain in darkness, and only insignificantly stronger in the presence of light (**Figure 4**).

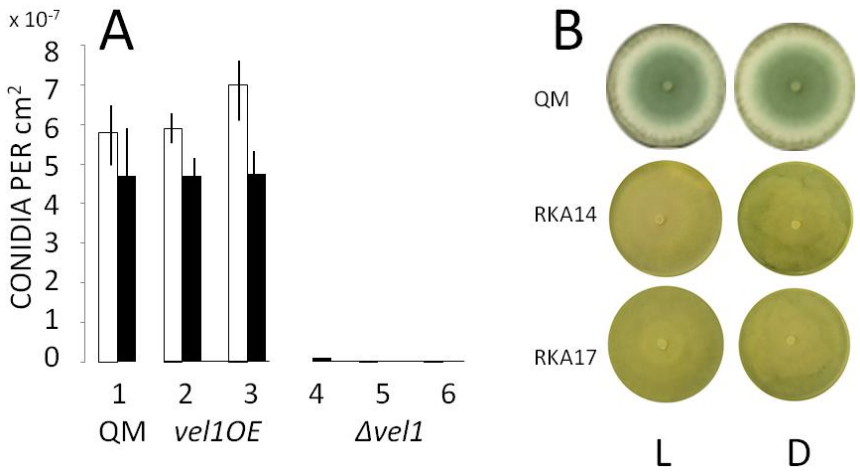

Fig. 4. Effect of *vel1* on asexual development of *T. reesei*. (A) Formation of conidia in the presence of ambient light (empty bars) and darkness (full bars) by *T. reesei* QM 9414, and three *Δvel1* and two *vel1*OE strains. Data are means of at least three independent biological experiments, and only results with $p < 0.05$ are shown. (B) Phenotype of the parent strain and two *Δvel1* strains after 5 days of growth on PDA.

In addition, sexual development of *T. reesei* (assayed by the formation of fertile perithecia) requires a functional VEL1 protein: the Δ*vel1* strain (*mat1-2*) did not produce any fruiting bodies when mated with the *T. reesei* tester strain CBS 999.79 (*mat1-1*; Seidl et al. 2009) in the presence of ambient light, whereas the parent strain QM 9414 did (**Figure 5**). Since fruiting body formation in *T. reesei* obligatorily requires light (Chen et al. 2010), the mating phenotype of *Δvel1* in darkness was the same as that of the parent strain. However, the *vel1*OE strain was able to form primordia also in darkness, although with less frequency than in light (**Figure 5C**). These primordia lacked asci and were thus not fertile (data not shown).

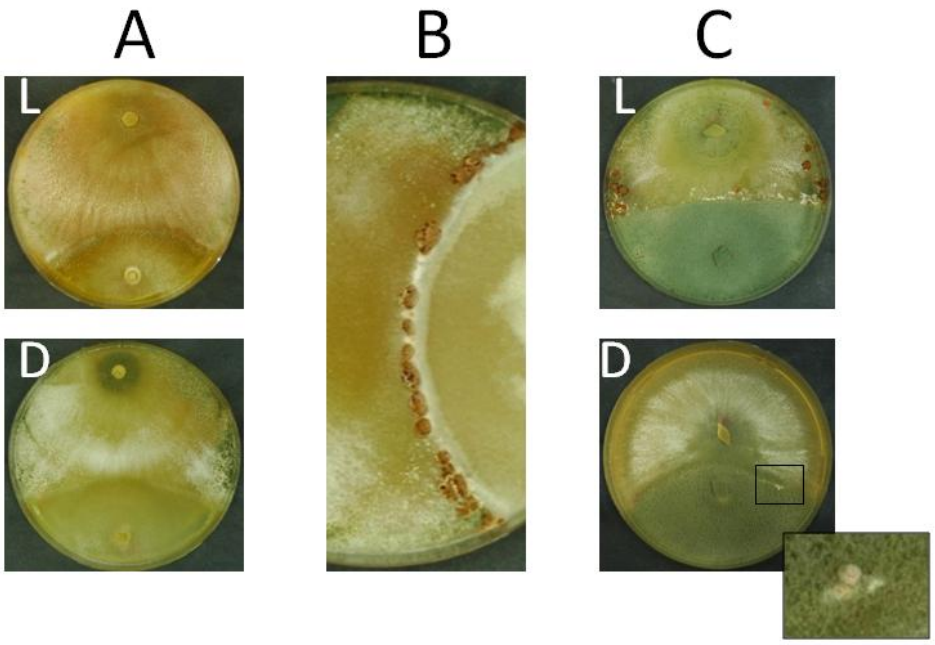

Fig. 5. VEL1 is required for sexual development in *T. reesei*. (A) Absence of fruiting body formation between *Δvel1* (top) and the compatible mating partner strain CBS 999.79 (bottom) in the presence of ambient light (L) and darkness (D). (B) Control plate between QM 9414 (left) and CBS 999.79 (right) in the presence of ambient light. (C) Fruiting body formation between *vel1*OE (bottom) and CBS 999.79 (top) under ambient light (L) or darkness (D). One of at least three replicate experiments is shown. The insert represents a magnification of the boxed part, highlightening the primordia.

VEL1 regulates cellulase and hemicellulase gene expression

Since we have previously shown that LAE1 regulates cellulase gene expression, we tested whether VEL1 would exert a similar effect. To this end, we cultivated the parent and mutant strains on lactose, a carbon source which induces cellulase expression, but whose utilization is independent of the action of secreted cellulases and therefore does not influence growth (Seiboth et al. 2007), and assayed expression of the two cellobiohydrolase genes *cel6A* and *cel7A*. The rationale for analyzing these two genes only was that the expression of the *T. reesei* cellulase genes and even of the transcriptional regulator *xyr1* is coregulated (Foreman et al. 2003, Seiboth et al. 2012, Portnoy et al. 2011, Ivanova et al. 2013). Growth of *the Δvel1* mutants on lactose was largely similar to that of the parent strain (maximally ± 15 % differences in biomass production). As shown in **Figure 6**, the Δ*vel1* strain expressed the two cellulase genes at about the same level, but the *vel1*OE strain massively overexpressed both cellulase genes at later stages of cultivation. In order to test whether this

increase in cellulase gene expression is dependent on the cross-talk between LAE1 and VEL1, we prepared a strain that overexpressed *vel1* in a *Δlae1* background (*vel1*OE*/Δlae1*). As shown in **Figure 6**, *cel6a* and *cel7a* gene expression were slightly reduced when compared to the parent strain, but not reduced to zero as in *Δlae1* (Seiboth Karimi et al. 2012).

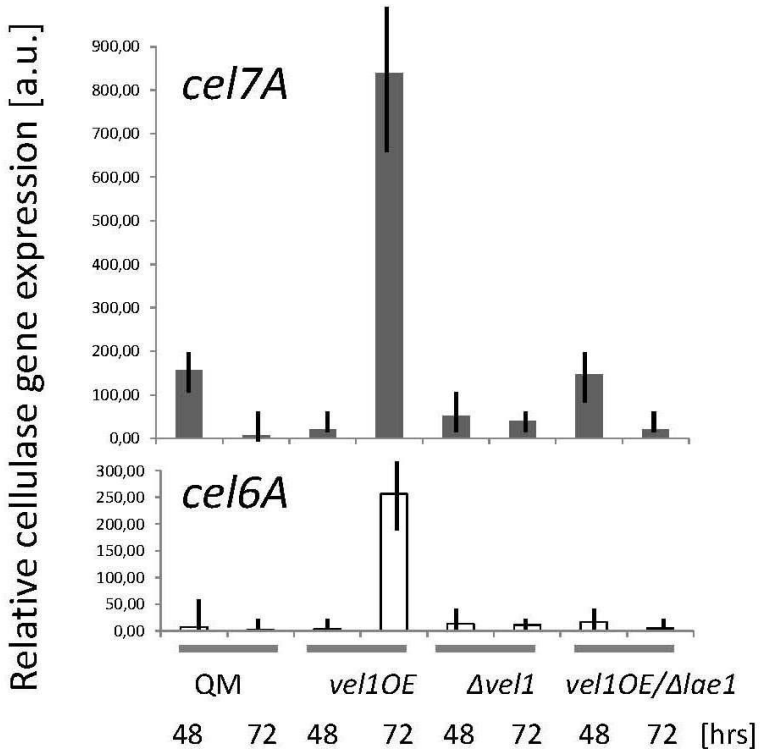

Fig. 6. Cellulase gene expression in *T. reesei* QM 9414 and its *vel1* mutants. Gene expression is given in arbitrary units, which were calculated by normalizing the *cel7A/tef1* and *cel6A/tef1* ratio to the lowest ratio observed (QM 9414, 72 hrs; set to 1). Data shown are means of at least two independent experiments and three measurements, and only results with $p < 0.05$ are shown.

To test whether this observation would also be reflected in the enzyme activities that the parent strain and the *vel1* mutants secrete into the medium, we cultivated the parent strain and several mutant strains on lactose, and cellulose and measured the extracellular cellulase and ß-glucosidase activities. Also cellobiohydrolases were also specifically assayed by the hydrolysis of 4-nitrophenyl-ß-D-cellobioside (Uzbas et al. 2012). The data shown in Table A2 (Apendix 4) support the results obtained by quantifying the expression of *cel7a* and *cel6a* (*vide supra*). The three *Δvel1*, two *vel1OE* and the two *vel1*OE*/Δlae1* strains behaved in a similar manner (data not shown).

These results prompted us to test whether the observed effect of VEL1 on cellulase formation would also apply to the formation of hemicellulases. To this end, we cultivated the parent and mutant strains on xylan, and measured the formation of xylanase and ß-xylosidase activity. Table A2 (Appendix 4) shows the respective results which indicate that overexpression of *vel1* also leads to the increased formation of these enzymes, whereas Δ*vel1* has no effect. Again, consistent data were obtained for different mutants (data not shown).

VEL1 overexpression stimulates *lae1* gene expression

The data described above suggest a stimulatory effect of VEL1 on cellulase gene expression that is not present in the absence of LAE1 function. We therefore tested by qPCR whether overexpression of *vel1* would effect *lae1* gene expression under cellulase producing conditions. Indeed, when the ratio of *lae1/tef1* expression in the parent strain was normalized to 1, the two *vel1:OE* strains RKA12 and RKA13 exhibited a ratio of 337 [± 76] and 584 [± 91] (two replicates, three measurements; 26 hrs of cultivation on lactose as a carbon source) respectively, thus indicating a massive upregulation of *lae1* in *vel1*OE.

Discussion

In this paper, we have explored the function of the *velvet* gene *vel1* of *T. reesei*. While this global regulator is conserved in numerous fungi, studies of *veA* orthologs across several fungal genera have now established that its impact on fungal development is quite versatile. As an example: conidiation is a trait influenced by velvet proteins from all fungi, but whereas a *veA/velA* knock out in *A. nidulans, P. chrysogenum* and *N. crassa* increases conidiation, it decreased conidiation in *veA/velA* knock-out mutants of *A. fumigatus*, *A. parasiticus*, *A. flavus, Fusarium fujikuroi*, *F. graminearum,*

Dothistroma septosporum and *T. virens* (Calvo et al. 2004, Krappmann et al. 2005, Amaike and Keller 2009, Wiemann et al. 2010, Hoff et al. 2010, Mukherjee and Kenerley 2010, Jiang et al. 2011, Chettri et al. 2012, Merhej et al. 2012, Park et al. 2012, Dhingra et al. 2012). *T. reesei* – like *T. virens* - belongs to the second group as the *vel1* knock-out strains almost completely lacked conidiation and this blockage was independent on the presence or absence of light. Asexual sporulation in a *vel1*OE strain occurred at the same level as in the parent strain, both in darkness and in light. These data indicate that conidiation in *T. reesei* is VEL1-dependent but unaffected by light. Interestingly, and unlike *T. virens* (Mukherjee and Kenerley 2010), we did not detect any enhanced formation of chlamydospores in the *vel1* knock-out strain.

As another example, we detected a complete loss of the ability of *T. reesei Δvel1* mutants to form fruiting bodies, which is in contrast to *N. crassa* (Bayram et al. 2008) but in agreement with reports on *A. nidulans* (Bayram and Braus 2012). Interestingly, the *vel1OE* strain enabled *T. reesei* to form some sterile primordia in darkness, an ability not shown by the wild type strains (Chen et al. 2012). This indicates that even a very high expression of *vel1* cannot overcome the inability of *T. reesei* to initiate fruiting body formation in the dark, but only initiates an early stage of fruiting. Thus the dependence of sexual development in *T. reesei* on light is independent of VEL1. Consequently, while we have shown that VEL1 also regulates developmental processes in *T. reesei*, its mode of action and its interaction with environmental triggers remains unclear and cannot be deduced from analogy with other fungal systems.

Also interesting, and in contrast to *A. nidulans*, *vel1* and *lae1* form a regulatory circuit: we have recently shown that *vel1* expression is dependent on the presence of *lae1* (Karimi-Aghcheh et al. 2013), and here we show that overexpression of *vel1* stimulates *lae1* expression. Overexpression of *lae1* has no effect on *vel1* transcription (R. Karimi-Aghcheh and C.P. Kubicek, unpublished data).

We have recently shown that LAE1 strongly impacts cellulase gene transcription in *T. reesei* and cellulase expression is completely abolished in *lae1* loss-of-function mutants (Seiboth Karimi et al.

2012). Since LAE1 and VEL1, as in other fungi, can physically interact in *T. reesei* (Karimi-Aghcheh et al. 2013), we assumed that cellulase expression would also be affected by *vel1*. In contrast, however, we did not see any significant reduction of cellulase transcription in the *Δvel1* mutants thus implying that cellulases are efficiently expressed even in the absence of VEL1. On the other hand, *vel1* overexpression under a constitutive promoter increased cellulase expression considerably and up to a level similar to that reached by *lae1* overexpressing strains (cf. Seiboth Karimi et al. 2012). This stimulation by VEL1, however, seems to require a functional *lae1* gene since a *Δlae1/vel1*OE strain expressed cellulases at a level comparable to that of the parent strain. We conclude from these findings that enhanced levels of VEL1 stimulate cellulase formation in *T. reesei* in the presence of a functional LAE1, but the absence of VEL1 function has no effect on cellulase gene expression. How do these findings relate to the current knowledge of physical or functional interaction of LAE1 and VEL1? *vel1* expression has been shown to be regulated by LAE1 (Karimi-Aghcheh et al. 2013), but this does not apply here because *vel1* was overexpressed under a promoter which is independent of LAE1 (*tef1*). LaeA was reported to prevent VeA in *A. nidulans* from conversion of the 63 kDa form into a 72 kDa form (Sarikaya Bayram et al. 2010), which seems to be partially due to hyperphosphorylation (Purschwitz et al. 2009), but it is not known if these findings also apply to *T. reesei*.

The present findings that a developmental regulator modulates cellulase gene transcription raises the question as whether cellulase formation would be linked to *T. reesei* development. In fact, we have recently shown that the transcription of cellulases and hemicellulases is strongly upregulated during *T. reesei* conidiation in a XYR1-dependent manner (Metz et al. 2011). However, the effect of VEL1 does not appear to be related to that: while the *Δvel1* strains are devoid in conidiation, they still form cellulases in amounts comparable to the parent strain. This is consistent with the findings that a *T. reesei Δxyr1* strain – which is completely unable to express its cellulase genes (Stricker et al. 2008) – sporulates normally (Metz et al. 2011). Likewise, cellulase formation seems to be unrelated to sexual reproduction, because *Δxyr1* strains of *T. reesei* still form fertile perithecia (L. Atanasova and

C.P. Kubicek, unpublished data). The hypothesis that formation of cellulases is necessary for *T. reesei* can therefore be rejected.

Cellulase formation has been reported to be stimulated by light, thereby depending on the function of the blue light receptor proteins BLR1, BLR2 and the LOV-PAS domain protein ENV1 (Schmoll et al. 2005, Castellanos et al. 2010, Schmoll 2011). In *A. nidulans*, the blue light receptors have been shown to interact with VeA, and one could therefore speculate that the effect of VEL1 on cellulase formation in *T. reesei* could be related to the observed modulation by light. However, under the conditions used in this study, cellulase formation is unaffected by loss-of-function of BLR1, BLR2 and ENV1 (Gyalai-Korpos et al. 2010) and their participation in the effect of *vel1* can therefore be ruled out.

A potential key to understanding of this stimulation of cellulase gene transcription in *vel1*OE strains may be our finding that *lae1* gene transcription is considerably increased in the *vel1*O*E* strain, at least under conditions of cellulase formation (i.e. on lactose). The actual stimulatory effect may therefore be due to an elicitation of the already established stimulation by LAE1 rather than VEL1 itself. Still VEL1 must be able to act on cellulase gene transcription also independently, because cellulase expression transcription is not reduced to zero in a *vel1*OE*/Δlae1* background (like in *Δlae1*; Seiboth Karimi et al. 2012) but maintained at the level of the parent strain. A possible explanation for this finding could be that under these conditions VEL1 can replace the function of LAE1 in cellulase regulation, but the mechanism of this remains obscure.

As a consequence, our previous findings of stimulation of cellulase gene expression by the overexpression of *lae1* cannot be interpreted as a result of the action of the VEL1/VEL2/LAE1 complex, but rather must be due to the action of LAE1 alone or in complex with proteins that have still to be identified. The fact that VEL1 can replace LAE1 in a Δ*lae1* strain is intriguing as it may indicate that the stimulation of cellulase gene expression by LAE1 is not directly due to the chromatin modifying activity of LAE1 (Reyes-Dominguez et al. 2010), but rather due to regulatory events that

can be individually effected by either LAE1 or VEL1. This is in accordance with our previous findings that there are no changes in histone 3A methylations on K3 and K9 the cellulase gene loci in *lae1* mutants (Seiboth Karimi et al. 2012, Karimi-Aghcheh et al. 2013). The essential role of LAE1 for cellulase gene expression thus remains enigmatic.

Materials and Methods

Strains and cultivation conditions

T. reesei strains used throughout this work are listed in **Table 1**. They were maintained on potato dextrose agar (PDA). *Escherichia coli* JM109 (Promega, Madison, Wisconsin) was used for plasmid construction and amplification. Cultures were grown at 28 °C in a Sanyo incubator containing a Philips-master light source (TLD-15 W/840), either with continuous illumination (1100 [± 30] lux, 30 cm distance) or in full darkness (dark conditions).

Table 1 Strains used in the present work

Strain name	Genotype	Reference
QM 9414	*mat1-2*	Seidl el al. 2009
CBS 999.79	*mat1-1*	
RKA14	*Δvel1/mat1-2*	This work
RKA12	*vel1OE, mat1-2*	
RKA13	*vel1OE, mat1-2*	
RKA15	*vel1OE/lae1Δ, mat1-2*	
RKA16	*vel1OE/lae1Δ, mat1-2*	
RKA17	*Δvel1/mat1-2*	
RKA18	*Δvel1/mat1-2*	

For cellulase production, *T. reesei* was grown in Mandels-Andreotti medium (Mandels and Andreotti 1978), using Avicel cellulose, lactose, beech wood xylan or glucose as a carbon source (1 %, w/v) as stated at the respective results.

Nucleic acid isolation and hybridization

Fungal mycelia were harvested by filtration, washed with distilled cold water, frozen and ground under liquid nitrogen. For extraction of genomic DNA, plasmid DNA and RNA, purification kits (Wizard Genomic DNA Purification Kit, PureYield Plasmid Midiprep System and RNeasy plant kit, respectively, all from Promega) were used according to the manufacturer's protocol. Standard methods were used for electrophoresis, blotting and hybridization of nucleic acids.

Construction of *T. reesei* recombinant strains

To study the function of VEL1, we constructed *T. reesei* strains in which *vel1* was deleted and strains, which *vel1* was expressed under the strong constitutive expression signals of the *tef1* (translation elongation factor 1-alpha encoding) promoter region (Akel et al. 2009).

To delete the *vel1* gene of *T. reesei,* the 1.8-kb *vel1* coding region was replaced by the *E. coli* hygromycin B phosphotransferase (*hph*) gene. This was performed by amplifying around 1.2-kb of the up- and downstream non-coding region of *vel1* from genomic DNA of *T. reesei* QM 9414 using the primer pairs given in Table A1 (Appendix 4)**.** The two resulting PCR fragments were digested with *Apa*I/*Xho*I (upstream region) and *Xho*I/*Cla*I (downstream region) and ligated into a *Apa*I/*Cla*I restricted vector pBluescript SK(+) (Stratagene, La Jolla, California), followed by the insertion of the 2.4-kb *Sal*I/*Xho*I fragment of the *hph* gene into the *Xho*I site resulting in pRKA_D122284hph.

For expression of *vel1* under a strong constitutive promoter, a 2,275-bp *vel1* PCR fragment including the coding and terminator region was amplified with the oligonucleotides Fw_ Ptef1:vel1_Cla1 and

tef1:vel1-HindIII [Table A1 (Appendix 4)] and then inserted downstream of the *tef1* promoter region (Akel et al. 2009) into the *Cla*I*/Hind*III sites of pLH1hphtef1 resulting in vector pRKA-OE122284hph, which contains the *E. coli* hygromycin B phosphotransferase (*hph*) under *T. reesei* expression signals as selection marker (Mach et al. 1994).

For knocking out *lae1* in the *vel1* overexpressing strain *T. reesei vel1*OE*,* it was co-transformed with pRKA_D41617pyr4 (7), and a 2.6 kbp fragment containing the *nptII* coding region under the 5' and 3' regulatory signals of the *A. nidulans trpC* gene amplified from pII99 (Namiki et al. 2001).

All vectors constructed were verified by nucleotide sequencing.

Fungal transformation

Protoplast preparation and DNA mediated transformation was performed as described (Guangtao et al. 2009). The strains were purified twice for mitotic stability, and integration of the expression cassettes was verified by PCR analysis. Gene copy numbers of the integrated constructs were determined by Southern analysis (Ausubel et al. 1999), using chromosomal DNA cleaved with *BamH*I/*Hind*III*.*

Analysis of sexual and asexual development

For sexual reproduction, *T. reesei* parent and mutant strains and the compatible mating partner strain CBS 999.79 (Seidl et al. 2009) were pre-grown on PDA for 4 days, and agar culture plugs then transferred on fresh PDA (Difco, Lawrence, KS, USA) on opposite sides of the plate at a 1 cm distance from the edge. The plates were kept at room-temperature and exposed to day light or kept in complete darkness for 4-7 days (see above). All pairs of strains which formed fruiting bodies were visually inspected until the maturation stage was achieved and ascospores were dispersed. Monoascospore cultures were isolated by dispersing the solution with a cotton swab on multiple PDA

plates. After overnight incubation several single germinated spores were selected with an aid of a stereomicroscope, transferred to a new PDA plate and cultivated at 28°C.

To test for photodependent conidiation, each PDA plate was inoculated with a 5-mm diameter mycelial plug taken from the edge of a 3-day-old colony. Three replications were done for each treatment. Plates were incubated at 28°C for 8 days in either complete darkness and cycles of 12h illumination/12 h darkness (see above), and conidia then harvested by gently rubbing them off in an equal volume of physiologically salt (0.1%, w/v, Tween and 0.8% w/v NaCl), filtering through glass wool, and centrifugation (5000 x g, 10 min). The conidia were then suspended in 2.5 g/l phytagel (Phytagel^{TM,} SIGMA, Steinheim, Germany), mixed and their transmission measured at 590 nm in a Biolog standard turbidimeter. The number of conidia was calculated using a calibration curve with *T. reesei* conidia.

Enzymatic assays and determination of fungal dry weight

Cellulase enzyme activities were determined using carboxymethylcellulose (1 %, w/v) and p-nitrophenyl-ß-D-cellobioside (Uzbas et al. 2012). Xylanases, ß-xylosidase and ß-glucosidase activities were determined as described previously (Kubicek et al. 1988). Protein in the culture supernatant was determined by the method of Bradford (1976). Fungal dry weight was determined by filtering an aliquot of the culture through glass sinter funnels (porosity G1), washing with tap water and drying at 80 °C to constant weight.

Analysis of gene expression by quantitative PCR

DNase treated (DNase I, RNase free; Fermentas) RNA (5µg) was reverse transcribed with the RevertAid™ First Strand cDNA Kit (Fermentas) according to the manufacturer's protocol with a combination of oligo-dT and random hexamer primers. All qPCR assays were performed on a Bio-Rad

(Hercules, CA) iCycler IQ. For the reaction the IQ SYBR Green Supermix (Bio-Rad, Hercules, CA) was prepared for 25 µl assays with standard $MgCl_2$ concentration (3 mM) and a final primer concentration of 100 nM each. All assays were carried out in 96-well plates. The amplification protocol consisted of an initial denaturation step (3 min at 95°C) followed by 40 cycles of denaturation (15 sec at 95°C), annealing [20 sec; for temperature see Table A1 (Appendix 4)] and elongation (10 sec at 72°C). Determination of the PCR efficiency was performed using triplicate reactions from a dilution series of cDNA (1; 0.1; 0.01; 0.001). Amplification efficiency was then calculated from the given slopes in the IQ5 Optical system Software v2.0. Expression ratios were calculated using REST© Software (Pfaffl et al. 2002). All samples were analyzed in at least two independent experiments with three replicates in each run.

Bioinformatic analysis

The *T. reesei* VEL1 protein sequence was used for a blastp search against the NCBI database what resulted in selection of 27 representative Pezizomycotina sequences. They were aligned by Clustal X version 2 (Larkin et al 2007), exported to GeneDoc 2.7 (www.psc.edu/biomed/genedoc).

Identification of PEST regions (protein domains that are enriched in proline, glutamic acid, serine, and threonine residues) that may lead to rapid protein degradation, typical for unstable proteins, was performed with epestfind (Rechsteiner et al. 1987, http://emboss.bioinformatics.nl/cgi-bin/emboss/epestfind). The predicted cellular localization of proteins was analyzed by WoLF PSORT (Protein Subcellular Localization Prediction tool; Horton et al. 2007, http://wolfpsort.org/), and leucine-rich nuclear export signals (NES) identified by NetNES 1.1 Server (la Cour et al. 2004, http://www.cbs.dtu.dk/services/NetNES/).

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Chapter 7

Summarizing Discussion

The subject of this thesis was the characterization of the functions of LAE1 in *Trichoderma reesei* with emphasis on cellulase regulation, as well as on the mycoparasitic properties of *Trichoderma atroviride*.

In 2004, LaeA was introduced first in *Aspergillus* by studies originally aimed to identify the genes influencing expression of aflatoxin biosynthesis (Bok and Keller 2004). Later on, the putative nuclear methyltransferase was characterized also as a global transcriptional regulator of secondary metabolism and virulence factor in other (pathogen) fungi (Wiemann et al. 2011, Wu et al. 2012, Kosalková et al. 2009, Bok et al. 2005). Moreover, it was shown that LaeA is also a part of the regulatory velvet complex of development and morphogenesis as well as SMs production in light-dependent manner in fungi (Bayram et al. 2008).

The orthologous *T. reesei lae1* gene was identified through a phylogeny approach (Seiboth, Karimi et al. 2012). Subsequent deletion of *lae1* gene in *T. reesei,* indeed, abolished total cellulase activity, whereas introducing more copies of *lae1* under control of a constitutive promoter *tef1*, into the *T. reesei* genome, elevated formation and thus expression of cellulase genes.

Towards understanding the mechanism of action of LAE1 in controlling the expression of cellulase gene clusters we hypothesized that, similar to *Aspergillus*, the targets of LAE1 action are subjected to LAE1-mediated counteraction of H3K9 methylation, and thus binding of HP1/HEP1 and formation of heterochromatin (Reyes-Dominguez et al. 2010). However, chromatin immunoprecipitation (ChIP) analysis of *Δlae1*, OE*lae1* and parent strain in CAZyme coding regions with antibodies, H3K4me2/H3K4me3 and H3K9me3 against active or silent chromatin, revealed that neither deletion of *lae1* nor its overexpression correlates with H3K4me2/H3K4me3 and H3K9me3, suggesting that modulation of H3K4 or H3K9 methylation is not the case for the action of LAE1 in regulating the expression of CAZyme genes in *T. reesi*. Also, in a genome wide analysis, only a very small portion of genome (=75 of 4089 genes of *T. reesei* bearing one or more of examined methylation marks) was affected by LAE1 action which was correlated with either H3K4me2 or H3K4me3 and thus implicating

that LAE1 does not directly alter the balance of an active (H3K4me2) and silencing (H3K9me3) histone modification under our conditions. Although more recently, an automethylation reaction done by LaeA was also proposed as possible mechanism of action of enzymatic reaction of LaeA (Panatananan et al. 2013) however, to date no biochemical evidence identifying the methyl-accepting substrate of LaeA as well as its direct effect on histone modification is available, therefore the precise function of that remains yet obscure and awaits further studies.

To gain more insight into the function of LAE1 in *T. reesei*, genome-wide expression of LAE1 mutants, Δ*lae1* and OE*lae1*, was assessed. Transcriptional profiles analysis of wild type, Δ*lae1* and OE*lae1*, showed in addition to cellulase genes reported earlier, genes encoding heterokaryon incompatibility proteins, ankyrinproteins, PTH11-receptors, 7 of 17 PKS or NRPS and oxidases/monoxygenases were major genes categories affected by LAE1.

T. atroviride is a widely used organism for studying mycoparasitism as well as regulation of conidiation by external environmental stimuli such as light and/or mechanical injury. In spite of seeing no difference in the sporulation pattern of *T. reesei lae1*-null-mutant in presence or absence of light, I could show that *T. atroviride* LAE1 is essential for asexual reproduction in darkness. Furthermore, in *T. atroviride* conidiation is stimulated by mechanical injury that has been already shown to be triggered by generation of radical oxygen species (ROS) after injury of mycelia (Casas-Flores et al. 2004, Steyaert et al. 2010, Hernández-Oñate et al. 2012). Complete loss of conidiation in *Δlae1* strain upon mechanical injury could not be explained by either oxidative stress (i.e. sensitivity to hydrogen peroxide) or formation of VOCs in *T. atroviride*, which have been shown to control sporulation in *T. atroviride* (Nemcovic et al. 2008, Carreras-Villaseňor et al. 2012) Similar to *C. heterostrophus,* in *T. atroviride*, increased sensitivity of *Δlae1* to oxidative stress provoked by H_2O_2 was observed, albeit being incomplete and occurred in higher concentrations than *C. heterostrophus* (Wu et al. 2012). Formation of VOCs reported also to stimulating conidiation in *T. atroviride* likely through providing signaling system for synchronization of conidation (Nemcovic et al. 2008, Carreras-

Villaseňor et al. 2012), however, VOCs from neither WT nor OE*lae1* could rescue reduced formation of conidia in *Δlae1* strain and therefore the hypothesis of indirect involvement of LAE1 through requirement of VeA for production of VOCs as reported for *A. parasiticus* is rejected (Roze et al. 2010).

Most importantly, *T. atroviride* LAE1 regulates positively the major trait of this fungus as being mycoparasitism of many plant pathogen fungi and thus its deletion reduced its ability to combat against other fungi. Loss of mycoparasitic ability of *T. atroviride* correlated with a significant loss in expression of several genes, including genes encoding cell wall hydrolases, secondary metabolites and proteins supposed to mediate hyphal contact to the host, which normally upregulated during mycoparasitism interaction and also led to partial reduction of formation of VOCs and WSC. Taken together these data and since reduced growth rate of *Δlae1* strain did not exceed ± 30 % of that of the parent strain under conditions of antagonism with the test fungi, thus attributing the effect of LAE1 on mycoparasitic ability of *T. atroviride* to growth defects due to loss of function of LAE1 is abandoned. Nevertheless, it's yet necessary to remind that - as shown in *T. reesei* - only few genes of secondary metabolism were affected by deletion of *lae1* gene while some of the secondary metabolism genes that have recently been shown to be upregulated during antagonism of *T. atroviride* against *R. solani* (Atanasova et al. 2013) were significantly downregulated in the *Δlae1* mutant suggesting divergent function of LAE1 in this genus which has been already reflected in mycoparasitic lifestyle of *T. atroviride* rather than saprophytic in *T. reeei*. However, it should also note that none of these genes exhibited enhanced expression in the OE*lae1* strain. However, our recent observation of the ability of *lae1OE* mutants in conversion of weak antagonistic activities of other *Trichoderma* spp. into vigorous mycoparasites (R.A. Karimi, M. Marzouk and I.S. Druzhinina, unpublished data) emphasizing on central action of LAE1 in mycoparasitism which awaits yet to be identified precisely.

As mentioned earlier, LaeA has been identified also as one of the major component of velvet complex regulating SMs metabolism as well as morphogenesis and development in all fungi investigated to date (Bayram et al. 2008, Wieman et al. 2011, Wu et al. 2012, Bayram et al. 2010, Hoff et al. 2010, Amaike et al. 2009). My data, that LAE1 is able to interact with VEL1 in Y2H analysis supports that this interaction also occurs in *T. reesei*. Interestingly, however, this interaction may not be absolutely necessary for cellulase regulation by LAE1: I found that deletion of *vel1* does not affect cellulase expression, whereas *vel1* overexpression stimulates it. I assume that this is due to the fact that overexpression of *vel1* leads to *lae1* upregulation. On the other hand, Δ*lae1/vel1*OE strain produced cellulases at the levels comparable to that of WT. Nevertheless, since cellulase transcription is not reduced to zero in a *vel1*OE/Δ*lae1* background - like Δ*lae1* (Seiboth, Karimi et al. 2012) - thus still, VEL1 must be able to act on cellulase gene transcription also independently by replacing the function of LAE1 in cellulase regulation through as yet unknown mechanism. Intriguing to mention that deletion of *vel1* gene caused complete loss of sporulation which is in good agreement with data obtained with *veA/vel1* knock-out mutants of some other fungi (Calvo et al. 2004, Wiemann et al. 2010, Mukherjee and Kenerley 2010, Jiang et al. 2011). Albeit recently-reported results on strong upregulation of (hemi) cellulases transcription during *T. reesei* conidiation in a XYR1-dependent manner (Metz et al. 2011) thus, since Δ*vel1* strains - despite being impaired in conidiation- expressed cellulases at the same level as WT, seems to be irrelevant to relate cellulase formation to asexual development of *T. reesei*. This is in contrast to Δ*lae1* strain impaired in formation of cellulases as well as asexual sporulation showed that about one third of conidiation genes that are upregulated during sporulation are downregulated in delta-*lae1* mutant and thus is in accordance to findings observed by Metz et al. 2011 (Seiboth, Karimi et al. 2012). Furthermore, similar to *A. nidulans,* deletion of *vel1* gene in *T. reesei* led to loss of fruiting bodies formation (sexual reproduction), however because *Δxyr1* strains of *T. reesei* still form fertile perithecia (L. Atanasova and C.P. Kubicek, unpublished data), likewise, attribution of cellulase expression to sexual

development is also rejected. Here it is yet worthwhile to mention that XYR1 is the main activator of major carbohydrate hydrolyzing genes such as *xyn1, xyn2, bxl1, cbh1, cbh2, egl1,* and *bgl1* of *T. reesei* (Mach-Aigner et al. 2008) and we have already shown that regulation of expression of cellulases by either LAE1 or XYR1 is dependent on the function of the other gene respectively (Seiboth, Karimi et al. 2012), and thus LAE1 and XYR1 regulate cellulase expression by independent pathways.

In Summary, data presented here show that specifically; LAE1 plays a key role in controlling major traits of *T. reesei* and *T. atroviride,* namely cellulase formation and mycoparasitism ability. Therefore, these data propose an attractive approach for enhancing cellulase production as well as biocontrol capacity in *T. reesei* and *T. atroviride* through single step strategy aimed for strain improvement. Moreover, both proteins, LAE1 and VEL1, have determining impacts on developmental processes of *Trichoderma*. However, notwithstanding growing body of evidences related to mentioned effects of LaeA/LAE1 and consequently VeA/VEL1 in various numbers of fungi, yet ongoing attempt to elucidate the mechanism of action of this regulatory system and identifying their possible other interacting partners in fungi and particularly in *Trichoderma* is necessary.

Chapter 8

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APPENDIX

APPENDIX 1

Table A1: CAZome genes that are upergulated in the *tef1:lae1* strain

protein ID	Identification	QM 9414 [log_2]	S.D. [log2]	*tef1:lae1* [log2]	S.D. [log2]	*tef1:lae1* (-fold)	p-value
3094	GH30 ß-1,6-glucanase	5.59588	0.12234	7.17447	0.6662	2.986 up	0.0223
5836	GH2 ß-mannosidase	10.7385	0.03373	11.79068	0.42011	2.073 up	0.0321
22129	GH61 celluilose monooxygenase CEL61B	10.91782	0.01727	12.59391	0.67915	3.195 up	0.041
27219	GH27 alpha-galactosidase	3.81507	0.46426	5.24526	0.10711	2.694 up	0.00533
41768	GH16 glycoside hydrolase	6.04602	0.26971	7.87103	0.65437	3.543 up	0.025
46816	GH3 ß-glucosidase	8.70765	0.07007	10.40969	0.23061	3.253 up	0.000655
47268	GH3 ß-glucosidase.	11.20929	0.0589	12.75641	0.28754	2.922 up	0.00411
49193	GH17 ß-glycosidase	12.94412	0.03577	13.9912	0.33158	2.066 up	0.0214
49976	GH45 endoglucanase CEL45=EGL5	8.40415	0.20013	13.33607	0.38552	30.525 up	0.000107
50215	GH16 ß-glycosidase, one transmembrane domain	11.3347	0.1033	12.58219	0.2964	2.374 up	0.000381
55802	GH76 alpha-1,6-mannanase	8.68349	0.01567	11.45617	0.65402	6.833 up	0.00589
55999	GH27 α-galactosidase	8.14024	0.03952	9.36371	0.24875	2.335 up	0.000868
56448	GH18 endochitinase CHI18-11	6.8206	0.09009	8.23413	0.10536	2.663 up	0.0000997
56894	GH18 endochitinase CHI18-10	2.87534	0.22714	4.25771	0.10343	2.606 up	0.00122
57179	GH88 glycosyl hydrolase	5.09815	0.27384	8.17708	0.56865	8.449 up	0.00268
58450	GH3 ß-xylosidase.	4.41688	0.14502	5.64895	0.31322	2.349 up	0.0129
58887	GH78 alpha-L-rhamnosidases	3.37525	0.1207	4.86028	0.11271	2.799 up	0.00046
59791	GH18 endochitinase CHI18-15	2.71721	0.04635	5.03302	0.41795	4.978 up	0.00233
60635	GH92 α-1,2-mannosidase	5.36203	0.26278	10.04624	1.90555	25.709 up	0.0186
65162	GH18 endochitinase CHI18-1b	10.93982	0.18638	13.36774	0.14046	5.381 up	0.000338
65380	1, 2-alpha-mannosidase	10.51249	0.12254	11.92898	0.25541	2.669 up	0.00339
69276	GH30 ß-glycoside hydrolase	7.51479	0.17067	11.80902	0.71743	19.619 up	0.000992
70186	GH28 polygalacturonase	5.05832	0.23144	6.71626	0.48761	3.155 up	0.00759
70845	GH55 ß-1,3-glucanase	9.03833	0.11074	11.58816	0.16389	5.855 up	0.00032
71245	GH18 endochitinase CHI18-1a	4.31858	0.19861	5.50133	0.43093	2.270 up	0.0356
71554	GH5 ß-glycosidase	3.70787	0.25126	5.34567	0.12291	3.111 up	0.000665
72071	CBM13 Carbohydrate-Binding Module Family 13	5.70232	0.3653	7.48403	0.61624	3.438 up	0.0249

72567	GH6 cellobiohydrolase CEL6A=CBH2	12.99037	0.15137	15.25272	0.15465	4.797 up	0.0000595
73005	GH79 ß-glycosidases	2.6602	0.01767	4.49246	0.09096	3.560 up	0.0000908
73101	GH16 ß-glycosidase	4.8095	0.11944	6.29322	0.1155	2.796 up	0.000564
73179	GH95 α-fucosidase	6.98958	0.06552	8.78224	0.42735	3.464 up	0.00832
73632	CE5 acetylxylan esterase AXE1	7.18943	0.14111	11.0991	0.28218	15.028 up	0.0000973
73638	CIP1	9.0132	0.17398	13.63062	0.37794	24.546 up	0.000107
73643	GH61 cellulose monooxygenase CEL61A	7.80786	0.10156	12.32581	0.15622	22.910 up	0.0000299
76210	GH62, abf2, a-L-arabinofuranosidase	7.01599	0.1865	10.47392	1.48912	10.988 up	0.00526
76672	ß-glucosidase 1 (EC 3.2.1.21)	7.04683	0.1593	11.84275	1.68676	27.778 up	0.0151
77299	GH2 exo-ß-D-glucosaminidase	8.93389	0.19956	10.1115	0.50636	2.262 up	0.00312
80340	GT32 α-mannosyltransferase	9.93872	0.1123	12.59314	0.15565	6.295 up	0.000242
81087	GH5 glycoside hydrolase	10.24147	0.0941	11.84336	0.55476	3.035 up	0.000925
82235	GH31 α-glucosidase B	12.75967	0.10122	14.26778	0.17921	2.844 up	0.00103
103049	GH28 polygalacturonase	5.31208	0.23593	6.61591	0.24359	2.468 up	0.00177
105448	CBM13 carbohydrate-binding protein	3.29043	0.21797	5.79799	0.39703	5.686 up	0.00229
108346	GH18 endochitinase CHI18-8	3.45563	0.07839	5.28355	0.25648	3.550 up	0.00141
108776	GH55 ß-1,3-glucanase	5.42228	0.13961	6.7659	0.23256	2.537 up	0.00257
109278	GH24 related to phage lysozymes	7.0068	0.14823	9.85215	0.82357	7.186 up	0.0163
110259	PL7 alginate lyase like protein	3.42471	0.11824	4.77698	0.11552	2.553 up	0.000728
110317	GH18 endochitinase CH18-17	3.11463	0.09064	4.81687	0.11685	3.254 up	0.000242
110848	protein with GH23 modules, glycoside hydrolase not yet assigned to a family	3.31933	0.16332	5.57066	0.5235	4.761 up	0.00532
110894	GH5 ß-1,4-endogalactanase	7.00883	0.12059	9.29206	0.9065	4.867 up	0.0145
111733	GH92 alpha-1,2-mannosidase	3.39345	0.32359	4.95686	0.10151	2.955 up	0.00107
111849	GH30 endo-ß-1,4-xylanase XYN4	9.07927	0.02793	10.11854	0.07768	2.055 up	0.000186
112140	GH28 polygalacturonase	4.07985	0.32754	8.3271	0.38039	18.991 up	0.00042
112392	GH11 candidate xylanase (XYL5)	4.89618	0.07513	6.92671	0.36806	4.085 up	0.00259
120229	GH10 xylanase XYN3	7.1759	0.11749	13.2519	0.8036	67.461 up	0.000643
120312	GH5 endoglucanase Cel5A	10.85671	0.20952	14.80739	0.26443	15.462 up	0.0000716
120749	GH1 CEL1A ß-glucosidase	13.19302	0.01401	14.50099	0.0573	2.475 up	0.000048

120873	GH71 α-1,3-glucanase, 2 CBM	4.82384	0.29231	8.28327	0.26109	11.000 up	0.000374
120961	GH61 endoglucanase CEL61B	8.6541	0.09645	13.06312	0.69301	21.244 up	0.000327
121127	GH3 BXL1 ß-xylosidase	10.11473	0.01254	12.3645	0.46332	4.756 up	0.000377
121746	GH55 exo-ß-1,3-glucosidase GLUC78	10.82838	0.07343	12.79041	0.48476	3.896 up	0.0105
122081	GH7 endoglucanase CEL7B=EGL1	8.68794	0.2276	13.75508	0.40052	33.524 up	0.000204
122780	GH28 polygalacturonase	11.30576	0.03609	13.91132	0.27322	6.086 up	0.000798
123232	GH12 endoglucanase Cel12A	10.75466	0.19955	14.31326	0.26129	11.782 up	0.0000743
123639	GH64 ß-1,3-glucanase	3.67615	0.23266	5.06862	0.24667	2.625 up	0.00331
123818	GH11 xylanase XYN2	11.69446	0.06929	13.01123	0.60738	2.491 up	0.000747
123940	methyl-glucuronoyl esterase CIP2	4.83643	0.37262	10.17903	1.68645	40.577 up	0.00715
123989	GH7, cellobiohydrolase Cel7A=CBH1	14.27507	0.04409	15.58843	0.08791	2.485 up	0.0000494
123992	swollenin	11.22247	0.1043	13.13211	0.65201	3.757 up	0.00312
124043	GH18 endochitinase CHI18-14	3.51068	0.38967	6.63093	0.22616	8.695 up	0.000612

The table shows the expression levels (in $\log_2$) of the lae1OE strain CPK4086 and QM 9414. “fold” indicates the enhancement in strain CPK4086;

Table A2. Genes that are >2-fold downregulated in delta-*lae1* >2-fold-upregulated during conidiation*

Protein ID	*Annotation*
70800	elastinolytic metalloproteinase
66551	AAA ATPase
46128	AAA+-type ATPase
69956	Alcohol dehydrogenase, class V
80659	alcohol oxidase AOX1
57940	alternative oxidase aox1
5970	Amidase
69863	Amidases
67806	Amino acid transporter
64971	Amino acid transporters
81420	Argonaute siRNA chaperone (ARC) complex subunit
70803	bifunctional catalase/peroxidase
59151	BYS1 domain protein
119826	C2H2 and homeobox transcription factor
112538	C2H2 transcription factor
111567	C2H2 transcription factor
120597	C2H2 transcriptional regulator (Egr2 ?)
4171	Ca2+ transporter
2316	caleosin related protein
64370	calpain-like protease
67538	Catalase
120371	Catalase
54219	CE acetyl xylan esterase
73632	CE acetyl xylan esterase AXE1
123659	cell wall protein. Distantly related to S. cerevisiae Pir3p.
73638	CIP1
67964	Cytochrome P450 CYP2 subfamily

64900	Cytochrome P450 CYP3/CYP5/CYP6/CYP9 subfamilies
59377	Cytochrome P450 CYP3/CYP5/CYP6/CYP9 subfamilies
66534	Cytochrome P450 CYP3/CYP5/CYP6/CYP9 subfamilies
75713	Cytochrome P450 CYP4/CYP19/CYP26 subfamilies
38749	Cytochrome P450 CYP4/CYP19/CYP26 subfamilies
52489	cytosin/purin permease
123946	dehydrogenase associated with cellulase signal transduction (PMID: 15288024)
70961	dienelactone hydrolase
65883	D-isomer-specific 2-hydroxy acid dehydrogenase
61553	D-xylulose 5-phosphate/D-fructose 6-phosphate phosphoketolase
82662	Epl1
61078	esterase/ lipase
106697	esterase/lipase, HGT
105823	FAD binding domain protein
66726	FAD binding protein
4114	FAD linked oxidase
104211	FKBP-type peptidyl-prolyl cis-trans isomerase
111716	Flavonol reductase/cinnamoyl-CoA reductase
111881	flavoprotein monooxygenases
72922	GABA permease, 12 TM
104222	GCN5-related acetyltransferase
112665	GCN5-related N-acetyltransferase
121418	G-D-S-L lipase/acylase
56840	GFO_IDH_MocA dehydrogenase
120749	GH1 CEL1A ß-glucosidase
120229	GH10 xylanase XYN3
123818	GH11 xylanase XYN2
123940	GH115 glucuronoyl esterase CIP2
123232	GH12 endoglucanase Cel12A
65333	GH15 candidate a-glycosidase (trehalase ?)

69245	GH2 ß-mannosidase
72632	GH27-α-galactosidase
103049	GH28: candidate polygalacturonase
121127	GH3 ß-xylosidase BXL1
69276	GH30 glucuronoxylan hydrolase
69944	GH31 a-glucosidase
4561	GH32, candidate a-glycosyltransferase
123226	GH37 trehalase
120312	GH5 endoglucanase Cel5A
81087	GH5 glycoside hydrolase
72567	GH6 cellobiohydrolase CEL6A
73643	GH61 cellulase activating protein CEL61A
120961	GH61 cellulase activating protein CEL61B
76210	GH62, abf2, a-L-arabinofuranosidase
72526	GH67 α-glucuronidase
122081	GH7 CEL7B endoglucanase 1
123989	GH7 cellobiohydrolase Cel7A
49081	GH74 xyloglucan hydrolase
71394	GH79 related to b-glycosidases
74198	GH92 α-1,2-mannosidase
5807	GH95 α-fucosidase
112022	glutathione-S-transferase
44366	Glycosylphosphatidylinositol-specific phospholipase C
72259	GPCR, RgsA-type
77547	GT1 glycosyltransferase
63756	helicase, DEAD-box superfamily
103189	HET protein
106171	HET protein
80142	HSP104 and related ATP-dependent Clp proteases
122363	Hsp26/Hsp42

46285	Hsp26/Hsp42
62100	Hsp30
55362	HSP70/HSC70, HSP70 superfamily
54352	hypothetical protein
123468	IMP dehydrogenase
76034	Iron/ascorbate family oxidoreductases
105968	Iron/ascorbate family oxidoreductases
105342	Iron/ascorbate family oxidoreductases
5182	iron-dependent peroxidase
77423	Jacalin-like lectin
105518	K+ channel
122820	Kynurenine aminotransferase, glutamine transaminase K
44175	lactate pyruvate transporter
55240	large-conductance mechanosensitive channel
104599	Mandelate racemase/muconate lactonizing enzyme
74194	mannitol dehydrogenase LXR1
58701	Mannose-6-phosphate isomerase
75165	MSF transporter
54972	MSF transpoorter
44956	MSF transporter
54036	MSF transporter
69957	MSF transporter
70933	MSF transporter
78585	MSF transporter
3405	MSF transporter
26642	MSF transporter
57749	MSF transporter
62380	MSF transporter (galactose permease?)
54239	Multicopper oxidases
44476	Multidrug resistance-associated protein, ABC superfamily

67971	MYND-type Zn-finger protein
76366	NADH:flavin oxidoreductase/12-oxophytodienoate reductase
60352	NADH-dehydrogenase (ubiquinone)
53079	NADP/FAD dependent oxidoreductase
109538	nZIP (ATF2 ?)
124097	phenazine biosynthesis PhzC/PhzF-type epimerase
124056	phosphatidic acid phosphatase
64959	phosphatidyl synthase
121498	Phosphatidylserine decarboxylase
123572	Phospholipase A2
67579	phospholipase A2
122824	PHT11-type GCPRs
73618	PKS
73621	PKS
82327	Pleiotropic drug resistance proteins (PDR1-15), ABC superfamily
82105	Pleiotropic drug resistance proteins (PDR1-15), ABC superfamily
111245	polysaccharide lyase; distantly related to chondroitin lyases
58418	Predicted unusual protein kinase
5647	PTH11-type GPCRs
47315	P-type ATPase
74282	QI74 orthologue
111053	retrograde regulation protein 2
72137	sceleton binding protein
59364	Sexual differentiation process protein ISP4
106164	short chain dehydrogenase/reductase
77202	short chain dehydrogenase/reductase
120911	short chain dehydrogenase/reductase
3055	short chain dehydrogenase/reductase
23090	short chain dehydrognease/reductase
34297	Stress responsive RCI peptide

106695	Sulfite oxidase, molybdopterin-binding component
76601	Sulfite oxidase, molybdopterin-binding component
123992	swollenin
70949	Thiamine pyrophosphate-requiring enzyme / benzoyl formate decarboxylase
103034	transcription factor 1-beta (cutinase)
2211	Transketolase
109276	Tripeptidyl peptidase II
80654	UDP-glucuronosyl and UDP-glucosyl transferase
107494	unique protein
123777	unique protein
124338	unique protein
123962	unique protein
120926	unique protein
103145	unique protein
105514	unique protein
120504	unique protein
121396	unique protein
122614	unique protein
111146	unique protein
121136	unique protein
110440	unique protein
70972	unique protein
105406	unique protein
124296	unique protein
105722	unique protein, HGT
105844	unique protein, secreted
74214	unknown protein
60422	unknown protein
112014	unknown protein
66092	unknown protein, only in fungi

124022	unknown protein
121416	unknown protein
68574	unknown protein
122089	unknown protein
111362	unknown protein
105287	unknown protein
109925	unknown protein
70894	unknown protein
60370	unknown protein
46764	unknown protein
104272	unknown protein
104295	unknown protein
124198	unknown protein
44967	unknown protein
60616	unknown protein
69375	unknown protein
119619	unknown protein
104174	unknown protein
60560	unknown protein
105237	unknown protein
60445	unknown protein
105455	unknown protein
109249	unknown protein
121439	unknown protein
121163	unknown protein
2033	unknown protein
123888	unknown protein
63868	unknown protein
103133	unknown protein
103031	unknown protein

123697	Unknown protein
54048	unknown protein
103576	unknown protein
42848	unknown protein
61526	unknown protein
75027	unknown protein
122792	unknown protein
82260	unknown protein
106686	unknown protein
58910	unknown protein
120623	unknown protein
4952	unknown protein
56853	unknown protein
61504	unknown protein
103060	unknown protein
23240	unknown protein
45598	unknown protein
103059	unknown protein
4851	unknown protein
79222	unknown protein
121486	unknown protein
105533	unknown protein
107853	unknown protein
58639	unknown protein
121653	unknown protein
81659	unknown protein
23228	unknown protein Duf1479
66696	unknown protein, Duf636
67133	unknown protein, 1TM
69483	unknown protein, 2 TM

82374	unknown protein, 3TM
50996	unknown protein, C2 domain
4626	unknown protein, C2 domain
110891	unknown protein, cupin region
54761	unknown protein, cupin region
5359	unknown protein, Duf1348
60810	unknown protein, GPR1/FUN34/yaaH protein, 6TMs
112239	unknown protein, secreted
33827	unknown protein, secreted
124282	unknown protein, SET and MYND domain
53824	unknown secreted protein
44965	zinc dependent alcohol dehydrogenase
65097	Zinc-binding oxidoreductase
23292	Zinc-binding oxidoreductase
112539	Zn2Cys6 transcription factor
120715	Zn2Cys6 transcription factor
70197	Zn-dependent hydrolase (beta-lactamase superfamily)

*strain CPK3793 was used; sporulation-upregulated genes were those described by Metz et al. (2011)

Table A3. LaeA/LAE1 protein sequences used to construct the phylogenetic tree and identify the *T.reesei* LAE1 orthologue

Fungal species	Protein ID*	Source**
Aspergillus fumigatus	XP_752835.1	http://www.ncbi.nlm.nih.gov/protein
Neosartorya fischeri	XP_001264291.1	http://www.ncbi.nlm.nih.gov/protein
Aspergillus clavatus	XP_001268793.1	http://www.ncbi.nlm.nih.gov/protein
Aspergillus parasiticus	AAX68414.1	http://www.ncbi.nlm.nih.gov/protein
Aspergillus flavus	AAX68412.1	http://www.ncbi.nlm.nih.gov/protein
Aspergillus nidulans	AAQ95166.1	http://www.ncbi.nlm.nih.gov/protein
Monascus pilosus	ABA87010.1	http://www.ncbi.nlm.nih.gov/protein
Aspergillus niger	XP_001389674.1	http://www.ncbi.nlm.nih.gov/protein
Aspergillus oryzae	XP_001819665.1	http://www.ncbi.nlm.nih.gov/protein
Aspergillus terreus	XP_001210764.1	http://www.ncbi.nlm.nih.gov/protein
Coccidioides immitis	XP_001243806.1	http://www.ncbi.nlm.nih.gov/protein
Pyrenophora tritici-repentis	XP_001934837.1	http://www.ncbi.nlm.nih.gov/protein
Phaeosphaeria nodorum	XP_001801609.1	http://www.ncbi.nlm.nih.gov/protein
Cochliobolus heterostrophus	91933	http://genome.jgi-psf.org/
Neurospora crassa	XP_965786	http://www.ncbi.nlm.nih.gov/protein
Neurospora discreta	96073	http://genome.jgi-psf.org/
Neurospora tetrasperma	38437	http://genome.jgi-psf.org/
Magnaporthe grisea	MGG_01233.6	http://www.broadinstitute.org/annotation/genome
Chaetomium globosum	CHGG_01690.1	http://www.broadinstitute.org/annotation/genome
Fusarium graminearum	FGSG_00657.3	http://www.broadinstitute.org/annotation/genome
Fusarium verticillioides	FVEG_00539.3	http://www.broadinstitute.org/annotation/genome
*Fusarium oxysporum 1****	FOXG_00975.2	http://www.broadinstitute.org/annotation/genome
*F. oxysporum 2****	FOXG_14806.2	http://www.broadinstitute.org/annotation/genome
Nectria haematococca	90349	http://genome.jgi-psf.org/
Trichoderma atroviride	42103	http://genome.jgi-psf.org/
Trichoderma virens	31676	http://genome.jgi-psf.org/

Trichoderma reesei	41617	http://genome.jgi-psf.org/

* protein IDs are preferentially given as those used in the NCBI database; when proteins are only present in genome databases, the respective protein ID used there is given

** wherever possible, the NCBI database was given priority; otherwise the respective genome databases are given

*** *F. oxysporum* contains 2 paralogues of LAE1, neither of which could be excluded and therefore both of them were used in the phylogenetic tree

Table A4. Oligonucleotide primers used in this study

Name	*Sequence (5'-3')
lae1 gene deletion	
5Trlae1Hind	TAAGCTTCACTCGCTTGTGTCTTC
5Trlae1Xho	TCTCGAGCGTTTATAGTGAGTAATGGC
3Trlae1Xho	TCTCGAGCTATTGCACTCTGTAAGCC
3Trlae1Apa	TGGGCCCTGGGTAGTGTTTCGTAATG
tef1-lae1 construction	
tef1Xhofw	GCCTCGAGGGACAGAATGTAC
ClaSalrv	AGTCGACATCGATGACGGTTTGTGTGATGTAGCGTG
TrLae1ATGCla	GCTATCGATGTCTCGAAACGCTCCCAAC
TrLae1TermHind	CGAAGCTTGCCCAAGGTCATCTTTCATTG
cDNA amplification	
LAE1ATG	GCCATGTCTCGAAACGCTC
LAE1stop	GCTTACAGAGTGCAATAGGC

*Respective restriction sites are underlined

Table A5. Primers for cellulase and *lae1* transcript auantification by Real Time PCR

Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
tef1 *	CCACATTGCCTGCAAGTTCGC	GTCGGTGAAAGCCTCAACGCAC
cel7a (cbh1)	CCGAGCTTGGTAGTTACTCTG	GGTAGCCTTCTTGAACTGAGT
cel6a (*cbh2*)	ACTACAACGGGTGGAACATTAC	CGTGGATGTACAGCTTCTCG
lae1	ACTGGAGATTGACTGGATGC	TTCTGCGTCTGGTAGCCTC

* *tef1* was used as a reference gene

APPENDIX 2

Table A1 Primers used for qPCR in this study

Gene	sequence (primer forward 5´→3´)	sequence (primer reverse 5´→3´)	primer efficiency (%)	Tm (°C)
Gene	primer efficiency (%)	Tm (°C)	100	57.2
vel1	AGCCTTATGTGCCTCACT	GCAGGAGCAGAGTAGTTG	100	57.2
lae1	ACTGGAGATTGACTGGATGC	TTCTGCGTCTGGTAGCCTC	100	62.9
Trire2:23171	GGTGGCTGTAGATGTGTT	TGAGAGTCGTCGTTGAAG	100	57.2
Trire2:112083	GGTGAAGAGATACGGTAT	TGAGTTGGAGTAGATGTC	100	52.6
Trire2:109101	GAATCCAGTGGCATTCTATC	CGACCCTCAATAATATCATACC	100	57.1
Trire2:69187	GCCTCTACCAATGAACTC	CCGAGACAGGACATAATC	97.2	56.1
Trire2:107071	CGATTGCCGTCTACGATAT	GCTCAGTTGTCCAGTCAT	100	56.1
Trire2:111494	ATCAACCAGACAGACAAC	AGATATAGACTTGCTCACATAG	95	52.6
Trire2:122824	CGTATGGCACCATCATTGG	CTGGGAAGACTCGTCGGAG	100	64
Trire2:39587	CTTGACAACCCGCCAAACAG	CGATGGAGTAAAAGACACCG	87.5	62
Trire2:82041	CTCATCGTCTTCCCCATTC	CATGCTTGTTGTTGTGCCCTG	100	62
Trire2:69904	CGGGAGACATTTCGCCTTTATC	CAGAAGCAGGTCGCAGAGTAG	86	64
Trire2:5647	CTACAACCCCGATAACCTC	CGTGGAAGATGCGGTAGTAC	98.5	60
Trire2:110744	CCTCTACGCCTCGTCAATC	CAATGGCGGAGAAGAAGAAGC	85	62
Trire2:110339	GATGCCGCTGATTGCCAC	CGAGATCGGTGATGATGTCG	91	65
Trire2:62462	CACGAACCCAACGCTCCAAC	CGAAGTATGCCCACTCCAG	95.5	63
Trire2:121990	CTCAAGGTAGTTGCGGTG	CGTCATCTGGTGGCTGTTG	90	60
Trire2:76763	CGGCAGAGGTTGAGCAATAC	CGGTAACACGGAGGCGTTTC	89	64
Trire2:105224	CACCGCTTGCCATCATCTTC	CAAGAGGAATCCCAAGACAC	100	61
sar1	TGGATCGTCAACTGGTTCTACGA	GCATGTGTAGCAACGTGGTCTTT	64	64

Table A2 A few genes are associated with H3K4 methylation, which would be consistent with a positive role LAE1 in H3K4 methylation*

protein ID	H3K4me2			H3K4me3			ratio *[Δlae1]*	ratio *[lae1OE]*	Annotation
	WT	*Δlae1*	*lae1OE*	WT	*Δlae1*	*lae1OE*			
47926	+	+	+	+			1,723 up	4,383 up	ABC1 family protein
72612			+				2,100 down	3,496 down	acetylornithine deacetylase
53372	+		+	+		+	2,753 down	2,231 down	acyltransferase 3
70859	+		+	+		+	4,320 down	5,344 down	amidase
81420	+	+	+	+			2,875 down	2,643 down	Argonaute siRNA chaperone (ARC) complex subunit
121785	+						19,861 down	2,194 down	ATP-dependent RNA helicase
65315	+		+				16,360 down	11,572 down	bZIP transcription factor
54703	+	+	+	+		+	2,647 down	2,513 down	C2H2 transcriptional regulator
102920	+	+	+	+		+	4,625 down	6,239 down	C2H2 transcriptional regulator
4876	+	+	+	+			2,534 down	5,033 down	catechol dioxygenase
123659	+		+	+		+	20,748 down	5,487 down	cell wall protein, istantly related to S. cerevisiae Pir3p.
75713	+	+	+	+		+	6,996 down	2,139 down	Cytochrome P450 CYP2 subfamily
120088	+	+	+	+		+	1,302 up	3,685 up	cytosolic asparaginyl-tRNA synthetase, required for protein synthesis, catalyzes the spe tRNA.
121877	+	+	+			+	4,705 down	3,710 down	epoxide hydrolase
79741	+	+	+	+		+	1,807 down	2,212 up	ER-bound Farnesyl-diphosphate farnesyltransferase
54144			+				1,097 down	3,141 up	ferric reductase
104211			+	+		+	15,509 down	24,344 up	FKBP-type peptidyl-prolyl cis-trans isomerase
2185			+				1,764 up	7,251 up	GCN5-related N-acetyltransferase
61703	+	+	+	+		+	1,316 up	2,241 up	germinal center kinase, related to S. cerevisiae Kic1
50215	+		+			+	6,380 down	2,374 up	GH16 endo-1,3-β-D-glucosidase/1,3-glucan binding protein
45717			+				4,496 down	4,485 down	GH47 α-1,2-mannosidase
82616	+		+				5,179 down	4,507 down	GH5 membrane bound endoglucanase CEL5b
82633			+				3,948 down	4,784 down	GH72 β-1 3-glucanosyltransferase
76620	+		+				2,041 down	2,566 down	Glycerol-3-phosphate dehydrogenase
123806	+						3,020 down	3,750 down	GPCR, secretin like

77557	+		+		+	7,316 down	5,660 down	GT glycosyltransferases not yet assigned to a family, 3 TMs
66888	+					11,913 down	5,286 down	GT α-1,3-mannosyltransferase
65817	+	+	+	+		5,911 down	3,410 down	GT α-1,3-mannosyltransferase CMT1
4561	+	+	+	+	+	2,718 down	2,569 down	GT α-1,6-mannosyltransferase
119963	+		+	+	+	1,202 up	2,031 up	HFBs
119805	+	+	+	+	+	1,232 up	2,096 up	HFBs
122363	+		+			2,768 down	2,497 up	Hsp26/Hsp42
105260	+	+	+	+	+	2,886 down	12,720 down	MFS permease
53475			+			2,083 down	2,851 down	MFS permease
68813			+			3,217 down	2,939 down	MFS permease
106248			+			1,696 up	3,534 up	monosaccharide transporter (galactose permease ?)
44476	+		+			4,980 down	2,986 down	MRP-type ABC transporter
73536			+			1,012 up	10,116 up	NADP-glutamate dehydrogenase
82105	+					2,755 down	4,288 down	PDR-type ABC transporters
64959	+	+		+	+	13,328 down	2,437 down	phosphatidyl synthase
3568	+	+	+	+	+	5,337 down	3,682 down	phospholipase A2
123572	+	+	+		+	2,634 down	2,278 down	Phospholipase A2
44278	+	+	+	+	+	2,647 down	4,813 down	Rab geranyl transferase escort protein
70355	+		+	+	+	5,994 down	18,605 down	SAM-dependent methylransferase
60758	+		+	+	+	12,719 down	5,079 down	SAM-dependent methyltransferase
4442	+					2,342 down	3,378 down	SAM-dependent methyltransferase
3055	+			+	+	22,757 down	2,485 down	short chain dehydrogenase/reductase
123616			+			1,984 up	2,365 up	short unique protein
71167	+		+			1,202 down	3,268 up	SSCRP
53366	+	+	+	+	+	1,353 up	2,151 up	translation initiation protein Sua5p.
106223	+	+	+	+	+	2,658 down	6,040 down	unique protein
119902	+	+	+	+	+	4,795 down	2,418 down	unique protein
43392	+		+		+	1,651 up	5,859 up	unique protein
104695	+	+	+	+	+	1,708 down	2,243 up	unique protein
105167			+			1,123 up	2,845 up	unique protein

107112			+			1,851 up	2,498 up	unique protein
4851	+		+	+	+	194,650 down	3,832 down	unknown protein
122089	+		+	+	+	7,444 down	3,053 down	unknown protein
65522	+		+			10,051 down	4,155 down	unknown protein
59940	+	+	+	+	+	3,732 down	3,082 down	unknown protein
60616	+	+	+	+	+	4,708 down	3,985 down	unknown protein
3488	+	+	+	+		2,876 down	2,578 down	unknown protein
107202			+			3,857 down	2,407 down	unknown protein
54622	+		+			2,553 down	4,686 up	unknown protein
43199	+		+			1,442 up	3,792 up	unknown protein
43302	+		+			1,007 down	5,446 up	unknown protein
71146	+		+			1,114 up	3,012 up	unknown protein
71154	+		+			1,158 up	2,392 up	unknown protein
120993	+					1,306 down	3,637 up	unknown protein
43083	+	+	+	+		1,236 down	3,216 up	unknown protein
105707			+			1,072 up	3,169 up	unknown protein
50996	+	+		+	+	4,528 down	7,482 down	unknown protein, C2 domain
49928			+			1,144 down	7,578 up	unknown protein, only in Sordariomycetes
102499			+			1,036 down	6,618 up	Zn2Cys6 transcriptional regulator

* genes are sorted alphabetically according to gene anniotation; other abbreviations and the color code are used as in Table A2

Table A3 Primers used for qPCR in this study

Gene	sequence (primer forward 5´→3´)	sequence (primer reverse 5´→3´)	primer efficiency (%)	Tm (°C)
Gene	**primer efficiency (%)**	**Tm (°C)**	100	57.2
vel1	AGCCTTATGTGCCTCACT	GCAGGAGCAGAGTAGTTG	100	57.2
lae1	ACTGGAGATTGACTGGATGC	TTCTGCGTCTGGTAGCCTC	100	62.9
Trire2:23171	GGTGGCTGTAGATGTGTT	TGAGAGTCGTCGTTGAAG	100	57.2
Trire2:112083	GGTGAAGAGATACGGTAT	TGAGTTGGAGTAGATGTC	100	52.6
Trire2:109101	GAATCCAGTGGCATTCTATC	CGACCCTCAATAATATCATACC	100	57.1
Trire2:69187	GCCTCTACCAATGAACTC	CCGAGACAGGACATAATC	97.2	56.1
Trire2:107071	CGATTGCCGTCTACGATAT	GCTCAGTTGTCCAGTCAT	100	56.1
Trire2:111494	ATCAACCAGACAGACAAC	AGATATAGACTTGCTCACATAG	95	52.6
Trire2:122824	CGTATGGCACCATCATTGG	CTGGGAAGACTCGTCGGAG	100	64
Trire2:39587	CTTGACAACCCGCCAAACAG	CGATGGAGTAAAAGACACCG	87.5	62
Trire2:82041	CTCATCGTCTTCCCCATTC	CATGCTTGTTGTTGTGCCCTG	100	62
Trire2:69904	CGGGAGACATTTCGCCTTTATC	CAGAAGCAGGTCGCAGAGTAG	86	64
Trire2:5647	CTACAACCCCGATAACCTC	CGTGGAAGATGCGGTAGTAC	98.5	60
Trire2:110744	CCTCTACGCCTCGTCAATC	CAATGGCGGAGAAGAAGAAGC	85	62
Trire2:110339	GATGCCGCTGATTGCCAC	CGAGATCGGTGATGATGTCG	91	65
Trire2:62462	CACGAACCCAACGCTCCAAC	CGAAGTATGCCCACTCCAG	95.5	63
Trire2:121990	CTCAAGGTAGTTGCGGTG	CGTCATCTGGTGGCTGTTG	90	60
Trire2:76763	CGGCAGAGGTTGAGCAATAC	CGGTAACACGGAGGCGTTTC	89	64
Trire2:105224	CACCGCTTGCCATCATCTTC	CAAGAGGAATCCCAAGACAC	100	61
sar1	TGGATCGTCAACTGGTTCTACGA	GCATGTGTAGCAACGTGGTCTTT	64	64

APPENDIX 3

Table A1: Primer used in this study

Triat2:	gene name or encoded protein	Primer name (forward)	Primer name (reverse)	sequence (forward)	sequence (reverse)	Primer efficiency	Tm (°C)
229937	*blr1*	Fw229937RTPCR	Rev229937RTPCR	AATGCCACCTCCAAGTCA	TTCTCGCTCTTCTCGTATTGA	100%	54
42429	*blr2*	Fw42429RTPCR	Rev42429RTPCR	GCCCTACCCTCATCAAAG	GCTTCTTCTTCTTCTCTCCT	100%	54
156014	GH16 ß-1,3/4-glucanase	RT_FW_156014 (0053)	RT_Rev_156014 (0053)	ATCTCGCAACACCTGGAA	TTCGGTTTCTCGGATGATATG	100%	58
134224	PKS	RT_FW_134224 (0057)	RT_FW_134224 (0057)	AGGGAACTGGAAACTTGA	ATGTAGCAGATGAGAGGATAA	100%	54,8
53332	SSCR protein	RT_FW_53332 (0059)	RT_Rev_53332 (0059)	AGCCGTATGTGGTGATAG	TTATTGTGCGGTAAGAGAA	94%	49,7
131539	SSCR protein	RT_FW_131539 (0060)	RT_Rev_131539 (0060)	CATTGCCTGTCTCCTCAA	ACCGCCATAGTTTCTCTTT	95,50%	54,8
145909	subtilisin-like protease	RT_FW_145909 (0066)	RT_Rev_145909 (0066)	GGCGTGGTTGTTAATGAG	AAATGGCAGCAAACAGAG	95%	497
85006	PKS	RT_FW_85006 (0058)	RT_Rev_85006 (0058)	TTGCCACAGAATCTTGAT	AGTCTTGATACACATTACCA	92,20%	52,5
144038	GH16 ß	RT_FW_144038 (0071)	RT_Rev_144038 (0071)	CCTACGATATTCACAGATG	TGTTGATGATAACCACTG	91,50%	52,5
128831	C-type lectin	RT_FW_128831 (0073)	RT_rev_128831 (0073)	GCATTGTTCATCTTGTAA	CTTCTCTTCGTAGGTATC	100%	52,5
34350	GH16 ß-1,3/1,4-glucanase	RTfw34350	RTrev34350	GTTCCACGAGAAGAATCCG	GCCTCCTCCACCAATGTC	93,50%	57,8
149951	Subtilisin-like protease	RTfw149951	RTrev149951	CTACCTACACTGCCACTC	AAGAAGAGTCGCTCAGAAT	90,90%	50,3
16930	aspartyl protease	RTfw160930	RTrev160930	AGGACTTTGAGGAGGGCAG	AGAAGCAGTGGCGTTGGA	100%	57,8
132795	C-type lectin	RT_atro_fw_ 132795	RT_atro_rev_ 132795	AGTTGGTCAATGTGATACG	CTGTCATTGTTGCTGGTA	91.6%	55,6
134073	cyanovirin_N	RT_FW_134073 (0076)	RT_Rev_134073 (0076)	TTGGCGGAGGAGCAAGTC	TTAATAGTTGAACACGAGAACCC	95.3%	54,8
42103	*lae1*	Lae1_ Atro_FW_RT	Lae1_ Atro_RV_RT	TATGAAGCAAGAGGATGT	AGTAAAGTAAGCGTGGTA	100%	
33350	lipoxygenase	Fw33350RTPCR	Rev33350RTPCR	TATCCAACGACACTCACAT	GGCATCCAGAGTCTTGAT	100%	54

APPENDIX 4

Table A1. Oligonucleotides used in the present work

	Forward primer		reverse primer	
Name	**sequence (primer forward 5´→3´)**	**Name**	**sequence (primer reverse 5´→3´)**	**Purpose**
Pfw_Dvel1_ApaI	TGGGCCCACCAGCAGCAGAAAGCAC	Prev_Dvel1_XhoI	TCTCGAGGATGCCGCGTTTGTTTCA	construction of *Δvel1*
Tfw_Dvel1_ XhoI	TCTCGAGGGCCATTGGACGAGTGTT	Dvel1_Trev1_ClaI	TATCGATTACCTATCGAGACTCATGG	construction of *Δvel1*
Fw_Ptef1:vel1_ClaI	GCTATCGATGGCGACGCCTTCCTCCGT	Tef1:vel1-HindIII	TAGTACCCCAAAGCTTTCAGGA	construction of *tef1:vel1* of *T.reesei*
RT_Vel1 (1200-End) fw	AGCCTTATGTGCCTCACT	RT_Vel1 (1200-End) rev	GCAGGAGCAGAGTAGTTG	Primers used for qPCR of *vel1*
cbh1 (Cel7A)_RT_Fw	CCGAGCTTGGTAGTTACTCTG	cbh1 (Cel7A)_RT_rev	GGTAGCCTTCTTGAACTGAGT	Primers used for qPCR of cel7A
cbh2 (Cel6A)_RT_Fw	ACTACAACGGGTGGAACATTAC	cbh2 (Cel6A)_RT_rev	CGTGGATGTACAGCTTCTCG	Primers used for qPCR of cel6A
RTfw-Lae1	ACTGGAGATTGACTGGATGC	RTrev-Lae1	TTCTGCGTCTGGTAGCCTC	Primers used for qPCR of lae*1*
Vel1-ATG	ACAAACGCGGCATCATGG	Vel1-stop	CAATGGCCTTACACCTGG	*vel1* probe for Southern blot
pVel1	: GCAAAAACGCACTGTAACTGG	hph_int	GTGTATTGACCGATTCCTTG	PCR Diagnosis of *Δvel1*
Vel_int1	CGACTACAATGCCAACTTC	Vel_int2	GTCGACGCATATACGGAAG	PCR diagnosis of *vel1* wild-type copy

Table A2. Formation of cellulase and hemicellulase enzyme activities by *T. reesei* QM 9414 (parent strain) and *vel1* mutant strains*

	Time	72 h				96 h				120 h			
enzyme	**carbon source**	**QM 9414**	***vel1OE***	***Δvel1***	***Δlae/vel1OE***	**QM 9414**	***vel1OE***	***Δvel1***	***Δlae/vel1OE***	**QM 9414**	***vel1OE***	***Δvel1***	***Δlae/vel1OE***
cellulase	cellulose	0,612 [± 0,082]	1,4 [± 0,132]	0,588 [± 0,073	0,614 [± 0,085]	1,35 [± 0,211]	2,6 [± 0, 224]	1,22 [± 0,182]	1,44 [± 0,082]	1,88 [± 0,47]	3,83 [± 0,67]	1,9 [± 0,22]	1,66 [± 0,37]
	lactose	0,431 [± 0,056]	1,07 [± 0,117]	0,408 [± 0,092]	0,44 [± 0,033]	0,622 [± 0,066]	1,68 [± 0,161]	0,612 [± 0,075]	0,678 [± 0,112]	1,22 [± 0,33]	2,78 [± 0,36]	1,33 [± 0,28]	1,28 [± 0,47]
cellobiohydrolase	cellulose	0,068 [± 0,022]	0,125 [± 0,027]	0,05 [± 0,004]	0,062 [± 0,017]	0,134 [± 0,022]	0, 217 [± 0,072]	0,158 [± 0,016]	0,118 [± 0,027]	0,22 [± 0,017]	0,352 [± 0,044]	0,25 [± 0,027]	0,24 [± 0,035]
	lactose	0,047 [± 0,012]	0,103 [± 0,023]	0,049 [± 0,009]	0,038 [± 0,010]	0,106 [± 0,042]	0,200 [± 0,051]	0,098 [± 0,038]	0,094 [± 0,009]	0,18 [± 0,019]	0,31 [± 0,015]	0,17 [± 0,012]	0,158 [± 0,023]
ß-glucosidase	cellulose	0,47 [± 0,035]	0,57 [± 0,04]	0,42 [± 0,062]	0,4 [± 0,032]	0,66 [±0,05]	1,45 [±0,07]	0,63 [±0,045]	0,71 [±0,072]	nd	nd	nd	nd
	lactose	0,273 [±0,04]	0,630 [±0,08]	0,281 [± 0,071]	0,245 [± 0,037]	0,571 [± 0,057]	1,23 [± 0,202]	0,61 [± 0,151]	0,588 [± 0,113]	nd	nd	nd	nd
ß-xylosidase	cellulose	0,215 [± 0,07]	0,53 [± 0,12]	0,201 [± 0,044]	0,22[± 0,022]	0,365 [± 0,07]	0,477 [± 0,08]	0,323 [± 0,07]	0,334 [± 0,08]	nd	nd	nd	nd
	xylan	0,455 [± 0,05]	0,72 [± 0,08]	0,43 [±0,51]	0,451 [± 0,019]	0,580 [± 0,09]	1,30 [± 0,10]	0,596 [± 0,09]	0,520 [± 0,10]	nd	nd	nd	nd
xylanase	cellulose	nd*	nd	nd	nd	0,36 [± 0,03]	0,487 [± 0,06]	0,33 [± 0,03]	0,310 [± 0,06]	0,37 [± 0,06]	0,585 [± 0,075]	0,34 [± 0,077]	0,30 [± 0,096]
	xylan	nd	nd	nd	nd	0,33 [± 0,03]	0,430 [± 0,07]	0,37 [± 0,03]	0,31 [± 0,07]	0,58 [± 0,09]	0,530 [± 0,045]	0,47 [± 0,059]	0,44 [± 0,075]

Values are means of 3-6 biological replicates and 2-4 independent measurements. Numbers in brackets indicate standard deviations. Note that only single mutants are shown in order to keep the table in a readable size; however, other mutants of the same type (delta or OE, respectively) produced consistent results.

* not determined

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Title of Thesis: *lae1*: a Regulator of *Trichoderma reesei* Secondary Metabolites
CGPA: A (sehr gut)
Supervisor: Univ. Prof. Dipl.-Ing. Dr. tech Christian P. Kubicek & Univ. Ass. Mag.rer.nat. Dr. Bernhard Seiboth

July 2004: **Master of Science M.Sc.** Biochemical Engineering

Place of Education: Amirkabir University of Technology (Tehran Polytechnic/Iran) www.aut.ac.ir
Title of Thesis: Xylitol Production from Iranian Sugar Cane Bagasse with the Yeast *Debaryomyces hansenii* CBS 767
CGPA: 17.50/20, **A**
Supervisor: Univ. Prof. Dr. Babak Bonakdarpour & Univ.Ass.Dr. Farzin Zokaee Ashtiani

July 2001: **Bachelor of Science B.Sc.** Applied Chemistry

Place of Education: Esfahan University www.ui.ac.ir
Title of Thesis: Analysis of Normal Paraffins by Subtractive Gas Chromatography & Kerosene-range of Normal Paraffin and Olefin Mixture by Gas Chromatography
CGPA: 14.99/20, **B**$^{+}$
Supervisor: Univ. Prof. Dr. Karim Movassaghi

Jun 1996: Natural Sciences **(Pre-university)**
CGPA: 18.37/20, **A**

Jun 1995: Natural Sciences (**High School (Diploma)**
CGPA: 18.96/20, **A**

Honors, Achievements & Awards

- **Austrian** Association for Middle East's (Österreichisches Orient- Gesellschaft Hammer-Purgstall Stipendium) Scholarship, 2008-2009
- **Ranked** 3th within the Graduate Students, Chemical Engineering Department of Amirkabir University of Technology, 2004
- **Ranked** 11 in the National Graduate Program Entrance Exam (Biochemical Engineering) out of about 400 Participants, 2001
- **Within** Top 2% of the Nation Wide Entrance Exam for Natural Sciences (ca. 120000 Participants), B.Sc. Programs, 1996
- **Within** Top 1% of Graduated Students of Pre-university, 1996
- **Top** Student among about 1000 High-School Students, 1995
- **Ranked** Second in the Laboratory Skills Competitions (Chemistry) among High School Students of the City of Esfahan, 1995
- **First** Place in Basketball Competitions among High Schools of the City of Esfahan, 1995
- **Marshal** Plan Scholarship, 2012.
- **Stipendium** für kurzfristige wissenschaftliche Arbeiten im Ausland, 2013

Professional Experiences

- **Laboratory** Assistant in Quality Control Department (Gas Chromatography Labs) of Iranian Chemicals Industry Investment Co. (LAB), Summer 2001
- **Bioethanol** Production from Hemicellulose Hydrolysate of Sugar Cane Bagasse with the Yeast "*Pichia stipitis"* (Research Assistant), Amirkabir University of Technology, (AUT), 2004
- **Collaboration** (as Consulting Engineer) with Chemical Industries Division of Karafarinan- e-Parnian Sanat Co., 2005-2007
- **Translator** of English Scientific Texts, Persica Office, 2006-2007
- **Project** Assistant in Molecular Biotechnology and Microbiology Groups of the Chemical Engineering Institute , Vienna University of Technology (since March, 2010)
- **Qualitative** Analysis of Peptaibols by Thin Layer Chromatography (TLC) from Fermentation Samples of AB- Enzymes Co. (Germany), Autumn, 2011

Work Shops & Membership

- **Upstream** Processing: Fermentation & Cell Culture; Cinna Gen Co, May 2006
- **International** Certificate in Production & Cultivation of Edible Mushrooms, Technical & Professional Skills Organization of Iran, 2007
- **qPCR** Seminar of Biorad Co. (16. September 2011, Vienna, Austria)
- Laser safety Workshop (Akademy der Seibersdorf Labor GmbH, 15.-16. 12.2011, Vienna, Austria)
- **Member** of Genetics Society of America (GSA), since November 2010
- **Training** in Michael Freitag laboratory (Oregon State University, USA), July 2012- February 2013 (ChIP and ChIP sequencing)
- **Certificate** of Confocal Microscopy training from Oregon State University (OSU), December 2012.
- **Training** in Levente Karaffa Laboratory (University of Debrecen), February 2013 until Mid of March 2013 (Fermentation)

Publications

- **Karimi-Aghcheh, R.,** L. Atanasova, B. Aquino, I. S. Druzhinina, and C. P. Kubicek (2013). **"The VELVET A orthologue VEL1 of *Trichoderma reesei* regulates development and acts as an auxiliary component of cellulase gene expression."** Submitted to Appl Microbiol Biotechnol (AMB).

- **Karimi-Aghcheh**, R., I. S. Druzhinina, and C. P. Kubicek (2013). "**The putative protein methyltransferase LAE1 of *Trichoderma atroviride* is a key regulator of asexual development and mycoparasitism."** Accepted in PLos ONE**.**

- **Karimi-Aghcheh**, R., J. W. Bok, P. A. Phatale, K. M. Smith, S. E. Baker, A. Lichius, M. Omann, S. Zeilinger, B. Seiboth, C. Rhee, N. P. Keller, M. Freitag, and C. P. Kubicek (2013). "**Functional Analyses of *Trichoderma reesei* LAE1 Reveal Conserved and Contrasting Roles of This Regulator. "** G3 (Bethesda) 3(2): 369-378.

- Seiboth, B., **R. Karimi Aghcheh**, P. A. Phatale, R. Linke, D. G. Sauer, K. M. Smith, S. E. Baker, M. Freitag and C. P. Kubicek (2012). **"The Putative Protein Methyltransferase LAE1 Controls Cellulase Gene Expression in *Trichoderma reesei."*** Molecular Microbiology. 84(6), 1150-1164.

- Degenkolb, T., **R. Karimi Aghcheh**, R. Dieckmann, T. Neuhof, S .E. Baker, I. S. Druzhinina, C. P. Kubicek, H. Brückner, H. Von Döhren (2011)."**The Production of Multiple Small Peptaibol Families by Single 14-module Peptide Synthetases in *Trichoderma/Hypocrea***." Chemistry and Biodiversity 9(3):499-535.

- **Karimi Aghcheh**, R., P. A. Phatale, K. M. Smith, S. E. Baker, J. W. Bok, B. Seiboth, N. P.Keller, M. Freitag and C. P. Kubicek (2012). " **Genome wide Insights into the Targets and Mechanism of Function of LAE1 in *Trichoderma reesei."*** 11th European Conference on Fungal Genetics (ECFG), Marburg, Germany, Poster.

- **Karimi Aghcheh, R.,** I. S. Druzhinina, and C. P. Kubicek (2012). "**Conodiation is the Major Target of LAE1 Function in *Trichoderma* a*troviride."*** 11th European Conference on Fungal Genetics (ECFG), Marburg, Germany, Poster.

- **Karimi Aghcheh**, R., J. W. Bok, M. Omann, S. Zeilinger, R. Linke, B. Seiboth, S. E. Baker, N. P. Keller, C. P. Kubicek (2011). "**The *Trichoderma* LaeA Orthologue LAE1 Identifies New Targets of Epigenetic Regulation in Fungi."** 26th Fungal Genetics Conference, Asilomar, California, USA, Poster.

- Von Döhren, H., T. Degenkolb, **R. Karimi Aghcheh**, R. Dieckmann, T. Neuhof, I. S. Druzhinina, C. P. Kubicek, S. E. Baker and H. Brückner (2011). **"Peptaibol Synthetases in *Hypocrea/Trichoderma*: Structures, Evolution and Production of Multiple Peptide Families."** 10th German Peptide Symposium (GPS), Berlin, Germany, Poster.

- Seiboth, B., **R. Karimi Aghcheh**, R. Linke, D. G. Sauer, S. E. Baker and C. P. Kubicek (2011). "**Epigenetic Regulation of Cellulase Gene Expression by *Trichoderma reesei."*** 6th Conference on Recombinant Protein Production, Vienna, Austria, Poster.

- **Karimi Aghcheh, R.**, B. Bonakdarpour and F. Zokaee Ashtiani (2005). "**Xylitol Production from Hemicellulose Sugar Cane Bagasse Hydrolysate with the Yeast *Debaryomyces hansenii* CBS767."** Proceedings of the 15th Iranian Food Industry Congress, Tehran, Iran, Oral Presentation.

- Christian P. Kubicek and **Razieh Karimi-Aghcheh.; Method for Enhancment of Protein Production by *Trichoderma reesei*. R61869** (**Patent**, acceptance in Process)