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MINI-REVIEW

Epigenetics as an emerging tool for improvement of fungal strains used in biotechnology

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Abstract Filamentous fungi are today a major source of industrial biotechnology for the production of primary and secondary metabolites, as well as enzymes and recombinant proteins. All of them have undergone extensive improvement strain programs, initially by classical mutagenesis and later on by genetic manipulation. Thereby, strategies to overcome rate-limiting or yield-reducing reactions included manipulating the expression of individual genes, their regulatory genes, and also their function. Yet, research of the last decade clearly showed that cells can also undergo heritable changes in gene expression that do not involve changes in the underlying DNA sequences (=epigenetics). This involves three levels of regulation: (i) DNA methylation, (ii) chromatin remodeling by histone modification, and (iii) RNA interference. The demonstration of the occurrence of these processes in fungal model organisms such as *Aspergillus nidulans* and *Neurospora crassa* has stimulated its recent investigation as a tool for strain improvement in industrially used fungi. This review describes the progress that has thereby been obtained.

Keywords Strain improvement · Filamentous fungi · Epigenetic · Secondary metabolites · Extracellular enzymes

Introduction

Fungi are used in many industrial processes, such as the production of primary and secondary metabolites, enzymes, vitamins, polysaccharides, pigments, lipids, and glycolipids, and others. Some of these products are produced commercially while others are potentially valuable in biotechnology. In addition to this, fungi are extremely useful in carrying out biotransformation processes which become increasingly important in biocatalysis and biorefinery (Rokem 2010).

The fungi used today for all these processes have undergone extensive improvement programs, because the originally isolated strains usually produced only minute or too low concentrations of the product of interest. Since all of the major industrial fungal species were isolated in their asexual forms, most of the strain improvement was done by classical mutagenesis and—after the advent of gene manipulation tools for fungi—by genetic manipulation. The latter strategies were more recently fortified by the development of reasonably priced tools for whole genome and RNA sequencing, combined with the development of high-throughput methods for genetic manipulation (Sharma 2015). This could overcome rate-limiting reactions by increasing the production of specific enzymes obtained by manipulation of the expression of the gene by gene amplification or the use of stronger promoters or altering the properties of the encoded protein by targeted mutagenesis. A complementary recent breakthrough in this area was the discovery of the presence of a cryptic sexual life cycle in several fungi including species used in industry (Böhm et al. 2013), which complements strain improvement programs by inbreeding techniques.

All the above-described strategies rely on the changing of the presence, expression, or structure of one or more genes in the respective genomes. Yet, research of the last decade has shown that cells can undergo heritable changes in gene expression (active versus inactive genes) that do not involve changes in the underlying DNA sequences. These changes in phenotype without a change in genotype have been named epigenetics, thereby making use of a term coined by

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Waddington (1942) as a portmanteau of the words epigenesis (a general theory first articulated by Aristoteles to describe the gradual and qualitative changes in development) and genetics. Although at the time of Waddington, the mechanisms of this phenomenon were, of course, unknown, his definition was supplanted over the years. In the last decade, epigenetic mechanisms have been most intensively investigated in areas like cell differentiation of eukaryotes (Bonasio 2015) and development and treatment of cancer and other diseases (Ptak and Petronis 2008). These studies revealed three major levels of epigenetic regulation: (i) DNA methylation, (ii) chromatin remodeling by histone modification, and (iii) RNA interference. The demonstration of the occurrence of epigenetic regulation and progress in understanding of its function in fungal model organisms such as *Aspergillus nidulans* and *Neurospora crassa* has recently stimulated its investigation also in industrially used fungi, particularly with respect to their performance and product formation.

In the present article, we will describe the recent advances in understanding of DNA methylation, chromatin remodeling, and RNA interference and, in filamentous fungi, how this information has been used for improvement of biotechnological processes and discuss what could be the next steps for further increases in the formation of the respective value-added products.

DNA methylation

DNA methylation is known to be essential for normal development and differentiation of plants and mammals (Reik and Dean 2001; Weber and Schübeler 2007; Jones and Baylin 2007). It occurs by the covalent addition of a methyl group at the 5-C of the cytosine ring resulting in 5-methylcytosine (5-mC) which extends into the major groove of DNA and thus inhibits transcription. In human DNA, 5-mC is found in approximately 1.5 % of genomic DNA. In somatic (but not in embryonic) cells, 5-mC occurs almost exclusively in the context of paired symmetrical methylation, a CpG site, in which a cytosine nucleotide is located next to a guanidine nucleotide. In the bulk of genomic DNA, most CpG sites are heavily methylated while CpG islands (sites of CpG clusters) in germ-line tissues and located near promoters of normal somatic cells remain unmethylated, thus allowing gene expression to occur. Methylation of a CpG island in the promoter region of a gene represses its transcription (Ho and Burggren 2010; Meyers 2012).

The occurrence and importance of DNA methylation in fungi are still unclear: DNA methylation has been observed in *Neurospora* and *Ascobolus* and some other filamentous fungi, while it has not found in many others, particularly in *Aspergillus* spp. In the truffle *Tuber melanosporum*, which has a genome with a particularly high (58 %) content of transposable elements and repetitive sequences, 5-mC was exclusively detected in the transposable elements but not the CpG islands (Chen et al. 2014). In *N. crassa*, it is found almost exclusively associated with relics of the genome defense system repeat-induced point (RIP) mutation (Selker et al. 1987). This is a gene silencing mechanism by which duplicated sequences (such as those from an invading transposon) are mutated during the sexual cycle, by littering each copy with C to T transition mutations (Cambareri et al. 1989; Selker 1990). In *N. crassa*, RIP occurs during the sexual stage in haploid nuclei after fertilization but prior to meiotic DNA replication (Selker et al. 1987). The resulting A/T-rich sequences are potent signals for de novo DNA methylation (Tamaru and Selker 2003). Genomic evidence indicates that RIP occurs or at least has occurred in the life history of most fungi including several *Aspergilli* (e.g., *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus fumigatus*, and *Aspergillus oryzae* (Clutterbuck et al. 2008)) and other biotechnologically relevant taxa such as *Trichoderma* and *Penicillium* (Kubicek et al. 2011; Braumann et al. 2008). In *Magnaporthe oryzae* and *Magnaporthe grisea*, Ikeda et al. (2013) detected mutations in DNA methyltransferases (see below) in a high portion of wild-type isolates and concluded that DNA methylation is on the way to be lost from this fungus. It is possible that this is also the case with other fungi, which would explain the partially conflicting results. Despite the view that DNA methylation is absent from yeasts, epigenetic changes in genome-wide DNA methylation were shown to be responsible for the ability of the flower-inhabiting yeast *Metschnikowia reukaufii* to exploit resources from a broad range of environments and were particularly important in harsh environments (Herrera et al. 2012).

The absence of DNA methylation has recently been revised in some fungi due to the availability of more sensitive techniques for analyzing DNA methylation (e.g., Tang et al. 2012). An alternative approach toward studying DNA methylation featured by many authors is the use of the DNA methylase inhibitor 5-azacytidine (5-AC). In *Aspergillus flavus* and *Aspergillus parasiticus*, two aflatoxin producers, formation of aflatoxins and asexual sporulation were strongly reduced upon addition of 5-AC (Yang et al. 2014a, b), although only negligible DNA methylation was found in *Aspergillus flavus* by the bisulfite sequencing method (Liu et al. 2012). A decrease in secondary metabolite production by addition of 5-AC has also been demonstrated in *Aspergillus clavatus* (Zutz et al. 2013) and the endophyte *Pestalotiopsis crassiuscula* (Yang et al. 2014a, b), whereas an increase was noted in other fungi (Chung et al. 2013; Liu et al. 2014). Results using inhibitors are always to be considered cautiously, however, because most of them bear side specificities. In the case of 5-AC, there is evidence that it is also incorporated into RNA, leading to destruction of nucleic acid and protein metabolism (Aimiuwu et al. 2012), and its inhibition of RNA methyltransferases has

also been discussed (Motorin et al. 2010). In addition, 5-AC is unstable in solution (Beisler 1978), and it is unclear whether this has been accounted for in the respective studies with fungi. In agreement with unspecific effects of 5-AC, Chen et al. (2014) showed that its addition only partially decreased DNA methylation in *Tuber melanosporum.*

With respect to the enzymes involved, two distinct DNA methyltransferases have been found in fungi: DIM-2 in *N. crassa* (Kouzminova and Selker 2001) and its ortholog Masc2 in *Ascobolus immersus* (Chernov et al. 1997) are involved in DNA methylation and transcriptional silencing in vegetative cells. DIM-2-dependent DNA methylation requires complex formation with heterochromatin protein 1 (HP1, the ortholog of *Schizosaccharomyces pombe* SWI6, a member of SWI/SNF family of ATP-dependent chromatin remodeling complexes; Freitag et al. 2004; Honda and Selker 2008; Honda et al. 2012).

The second DNA methyltransferase is Masc1 of *Ascobolus immerses*. It is responsible for development and premeiotically induced DNA methylation during the sexual stage (Malagnac et al. 1997). Its *N. crassa* ortholog RID is required for repeat-induced point (RIP) mutation during the sexual phase (Freitag et al. 2002).

While DNA methylation could, so far, not be detected, e.g., in *Aspergillus nidulans* and other *Aspergillus* spp. (Tamame et al. 1983; Montanini et al. 2014), a DNA methyltransferase ortholog of *N. crassa* RID and *Ascobolus immersus* Masc1 (DmtA) was still detected in *Aspergillus nidulans* and is essential for sexual development. Yet, DNA methylation or MIP could not be demonstrated (Lee et al. 2008). A phylogenetic analysis shows that within the *Pezizomycota*, two DNA methyltransferases (typified by *N. crassa* DIM-2 and RID) have evolved in the *Sordariomycetes*, whereas the DIM-2-like protein has been lost in the *Aspergilli* although not in basal members of the *Eurotiales* (e.g., *Coccidioides*, *Uncinocarpus*, etc.; Fig. 1). Yet, orthologs of the above two DNA methyltransferases appear to be absent from genomes of *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and *Caenorhabditis elegans* (Antequera et al. 1984; Proffitt et al. 1984; Hodgkin 1994), which correlates with the absence of methylated DNA in these organisms.

Examples of regulation of gene expression by DNA methyltransferases in fungi are rare, but, e.g., in *N. crassa*, sequences within the promoter of *frequency* (*frq*), a negative element of the circadian clock (Baker et al. 2011), are methylated in a DIM-2-dependent way.

DNA methylation is generally considered to be rather stable, but the methyl group must obviously be removed for epigenetic reprogramming. This can occur either passively or actively or by a combination of both. While passive DNA demethylation usually takes place on newly synthesized DNA strands during replication rounds, active DNA demethylation starts with an oxidative modification of cytosine bases by the 2-oxoglutarate-dependent cytosine dioxygenase enzymes of the ten eleven translocation (TET) family. The oxidized cytosine may then be either completely removed during replication or undergo further enzymatic degradation (Wu and Zhang 2011). This process has not yet been studied in fungi, however, and a BLASTP search with the human TET proteins against several genome databases of filamentous fungi did not produce any hits (R. Karimi Aghcheh and C.P. Kubicek, unpublished data).

Chromatin modification

Chromatin is the complex of DNA and proteins that are packed within the nucleus of eukaryotic cells. To form chromatin, tightly condensed DNA wraps around histones to form the nucleosome, which consists of 146 base pairs of double-stranded DNA and eight histone proteins. This tightly packed chromatin, which is thus not accessible for transcription to occur, is termed heterochromatin, whereas the loosely packed and transcriptionally accessible form is called euchromatin. The genomic inventory of the basic chromatin components in different filamentous fungi has been recently reviewed, and chromatin regulation of several catabolic genes was summarized by Brosch et al. (2008) and García et al. (2008). The Histones can undergo posttranslational modifications that alter their interaction with DNA and nuclear proteins. Particularly, the H3 and H4 histones have long tails protruding from the nucleosome, which can be covalently modified at several places, through methylation, acetylation, phosphorylation, ubiquitination, SUMOylation, citrullination, and ADP-ribosylation (Campos and Reinberg 2009). The combination of different modifications is considered to be the “histone code” which is typical of specific conditions (Strahl and Allis 2000).

Investigations on the role of histone modifications in chromatin regulation done in the two model fungi *N. crassa* and *Aspergillus nidulans* showed that methylation of lysine (K) and arginine (R) and acetylation of K residues constitute the main tools used. The N-terminal tails of H3 and H4 are required to generate transcriptionally repressive heterochromatin and transcriptionally active euchromatin. Typically, in euchromatin, the K residues in the H3 and H4 tails are hyperacetylated, and especially, H3K4 is trimethylated. In heterochromatin, on the other hand, H3K9 is trimethylated and other K residues are hypoacetylated (Noma et al. 2001). Several K residues have been also identified as targets for acetylation and methylation in filamentous fungi (Table 1).

A plethora of proteins participates in histone modifications. Genes encoding the enzymes performing the reactions outlined earlier (i.e., histone acetyltransferases (HATs), histone deacetylases (HDACs), SET-domain-containing histone methyltransferase (HMT) proteins with Jumonji domains,

protein arginine methyltransferases (PRMTs), kinases, phosphatases, and ubiquitin ligase-containing proteins; cf. Rando and Winston 2012) have all been identified in the genomes from filamentous fungi. Table 1 contains a list of histone-modifying enzymes and their already characterized amino acid targets.

The specific histone modifications described above serve to recruit also other proteins by specific recognition via specialized protein domains. For instance, proteins with coactivator or other transcription-related functions such as bromodomain proteins have been shown to be part of the complexes that can recognize acetylated K residues (Sterner and Berger 2000). Methylated K residues, on the other hand, bind chromodomain proteins that—depending on the methylation type (see Table 1)—can form either transcriptionally silent heterochromatin (e.g., the heterochromatin proteins HP1/HepA [Freitag et al. 2004; Reyes-Dominguez et al. 2010]) or result in transcriptional activation (e.g., chromodomain helicase DNA-binding [CHD1], an ATP-dependent chromatin remodeling enzymes required for normal circadian regulated gene expression of the central clock gene *frequency*) (Belden et al. 2007; Belden et al. 2011).

In fungi, the most enigmatic protein which affects chromatin modifications is perhaps the methyltransferase domain protein LaeA/LAE1 (loss of *aflR* expression A) that directly interacts with transcription factors of the Velvet domain family (Bok and Keller 2004; Bayram et al. 2008). LaeA was originally identified in *Aspergillus nidulans* to control transcription of several secondary metabolite synthesis gene clusters (Bok and Keller 2004). This protein contains no obvious DNA-binding motif but domains characteristic of arginine protein methyltransferases and a seven-beta-strand containing S-adenosyl-L-methionine (SAM)-binding domain (Bok and Keller 2004). This led to initial hypothesis that LaeA may control secondary metabolism gene expression through its methyltransferase activity that could act on histones. However, so far, *Aspergillus nidulans* LaeA was found to perform only an automethylation reaction at methionine-207 which is close to the adenosylmethionine-binding site, and the exchange of methionine-207 did not lead to a loss of function of LaeA (Patananan et al. 2013). Its function must thus occur in another context. In this regard, it was demonstrated that LaeA directly interacts in the nucleus with transcription factors of the trimeric Velvet complex consisting of the Velvet domain proteins VeA and VelB, in which VeA interacts with LaeA (Bayram et al. 2008). The *Aspergillus nidulans* Velvet complex coordinates fungal development and secondary metabolism in response to a number of environmental stimuli (Bayram et al. 2008; Bayram and Braus 2011). Interestingly, VeA can interact not only with the LaeA, but also with at least three other methyltransferases: the LaeA-like methyltransferase F (*limF*), and the methyltransferase heterodimers VipC–VapB. Both are negative regulators of sexual and positive

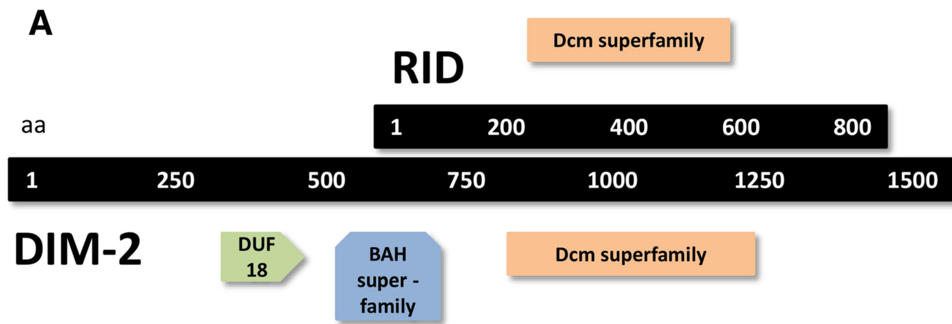
Fig. 1 DNA methyltransferases in filamentous fungi. **a** Protein structure of DIM-2 and RID from *Neurospora crassa*. *BAH* bromo-adjacent homology domain, *Dcm* site-specific DNA methylase (DNA replication, recombination, and repair). **b** Phylogenetic analysis of two DNA methyltransferases, DIM-2 and RID (typified by *N. crassa* DIM-2 and RID), within *Pezizomycota*. DIM-2 and RID have evolved in the *Sordariomycetes* (*lilac rectangle*), whereas the DIM-2-like protein has been lost in the *Aspergilli* (located *inside* the *green rectangle*). The tree was constructed by neighbor Joining in MEGA 5.0 (Tamura et al. 2011) with 1000 bootstrap replicates (coefficients are indicated below the respective nodes). Gaps in the alignment were not considered

regulators of asexual development and act in the opposite way of LaeA (Sarikaya-Bayram et al. 2015). However, they all are not localized to the nucleus and, therefore, are likely not involved in histone modification.

Histone SUMOylation is associated with transcriptional repression via modification of the histones H4, H2A, and H2B by small ubiquitin-related modifier (SUMO) family proteins (Nathan et al. 2006; Shiio and Eisenman 2003). Recently, a link between the complex network of the SUMO and velvet domain complex was identified in *Aspergillus nidulans* (Harting et al. 2013). This network encompasses a large number of interacting proteins including those involved in histone modifications or transcription. One of them is CclA, a subunit of the COMPASS complex and homolog of the yeast Bre2 which is responsible for methylation of H3K4 (Bok et al. 2009; Wood et al. 2005), and other proteins incorporated or interacting with the chromatin-modifying SAGA complex. Another one is RcoA, an ortholog of the general transcriptional repressor of yeast TUP1, and a regulator in *Aspergillus nidulans* sexual development that acts downstream of *veA* (Todd et al. 2006). RcoA thus is a candidate for the link between the SUMO network and the velvet domain complex and, thus, the involvement of the velvet complex at the level of chromatin modification.

RNA interference

Non-coding RNAs (ncRNAs) are functional RNA molecules that are transcribed from DNA yet are not translated into proteins but, instead, regulate gene expression at the transcriptional and posttranscriptional level. They can be divided into two main groups: short ncRNAs (with a length of less than 30 nts) and long ncRNAs (generally longer than 200 nts), the former comprising microRNAs (miRNAs), short interfering RNAs (siRNAs), and piwi-interacting RNAs (piRNAs). miRNAs and siRNAs bind to specific target messenger RNAs (mRNAs) with a complementary sequence and inhibit translation by inducing its degradation. siRNAs function in a similar way as miRNAs by mediating posttranscriptional gene silencing as a result of mRNA degradation. piRNAs acquired their name due to association of these siRNAs with proteins


A
RID
Dcm superfamily
aa
1 200 400 600 800
1 250 500 750 1000 1250 1500
DIM-2
DUF 18
BAH super - family
Dcm superfamily

B
Neurospora crassa CAC10091.2
Neurospora crassa AF500227.1
Neurospora intermedia AF500228.1
Sordaria macrospora CAJ40944.1
Myceliophthora thermophile AEO54902.1
Podospora anserine XP001907409.1
Thielavia terrestris AEO67982.1
Chaetomium thermophilum CTHT 0012460
Colletotrichum graminicola EFQ26585.1
Eutypa lata XP007790886.1
Magnaporthe oryzae EHA46203.1
Magnaporthe oryzae XP003720946.1
Beauveria bassiana EJP68360.1
Metarhizium album KHN98759.1
Acremonium chrysogenum KFH43323.1
Stachybotrys chartarum S40288.04352
Trichoderma reesei AEM66210.1
Fusarium graminearum FGSG08648
Nectria haematococca EEU46991.1
Fusarium verticillioides EWG38988.1
Fusarium oxysporum EWZ40072.1
Fusarium oxysporum ENH61904.1
Sordariomycetes
Ascobolus immerses AF025475.1
RID
Blastomyces dermatitidis EEQ70330.1
Histoplasma capsulatum EGC49841.1
Paracoccidioides brasiliensis XP010760344.1
Coccidioides immitis EAS27225.2
Uncinocarpus reesii
Microsporum gypseum EFQ97221.1
Trichophyton rubrum EZF10319.1
Talaromyces stipitatus XP002479658.1
Penicillium expansum KGO49141.1
Penicillium roqueforti CDM37374.1
Aspergillus oryzae XP001823049.2
Aspergillus nidulans AF428247.1
Aspergillus clavatus XP001270179.
Aspergillus fumigatus EDP47610.1
Neosartoryafischeri XP001257580.1
Eurotiomycetes
DmtA
Trichophyton tonsurans EGE00804.1
Coccidioides immitis EAS36569.2
Paracoccidioides brasiliensis EEH48849.2
Eurotiomycetes
Botrytis cinerea CCD50629.1
Myceliophthora thermophile MYCTH2095291
Thielavia terrestris AEO67982.1
Chaetomium globosum EAQ83224.1
Neurospora crassa AF348971.1
Sordaria macrospora CAJ40945.1
Eutypa lata EMR67180.1
Magnaporthe oryzae EHA48492.1
Podospora anserine CAP64635.1
Colletotrichum gloeosporioides CGLO13038
Beauveria bassiana EJP66406.1
Cordyceps militaris EGX92458.1
Metarhizium album KHN97336.1
Acremonium chrysogenum KFH48237.1
Stachybotrys chartarum KFA79069.1
Trichoderma reesei ETS02468.1
Nectria haematococca EEU42518.1
Fusarium graminearum ESU18016.1
Fusarium verticillioides EWG52814.1
Fusarium oxysporum EXA39217.1
Sordariomycetes
DIM-2
0.2

Table 1 Histone codes and histone-modifying enzymes from fungi

	Modified site	Modification pattern	Modifying enzyme	Proposed function	References
H1	–	–	–	–	–
H2A	K4 (*S. cerevisiae*)	Acetylation	Esa 1	Transcriptional activation	Clarke et al. (1999)
	K7 (*S. cerevisiae*)	Acetylation	Esa1	Transcriptional activation	Clarke et al. (1999)
	K126 (*S. cerevisiae*)	SUMOylation	Ubc9	Transcriptional repression	Nathan et al. (2006)
H2B	K11 (*S. cerevisiae*)	Acetylation	Gcn5	Transcriptional activation	Suka et al. (2001)
	K16 (*S. cerevisiae*)	Acetylation	Gcn5, Esa1	Transcriptional activation	Suka et al. (2001)
	K123 (*S. cerevisiae*)	Ubiquitylation	Rad6	Transcriptional activation	Robzyk et al. (2000)
	K6 or K7 (*S. cerevisiae*)	SUMOylation	Ubc9	Transcriptional repression	Nathan et al. (2006)
H3	K4 (*S. cerevisiae*)	Acetylation	Esa1	Transcriptional activation	Clarke et al. (1999)
	K14 (*S. cerevisiae*)	Acetylation	Esa1	Transcriptional activation	Clarke et al. (1999)
	K56 (*S. cerevisiae*)	Acetylation	Spt10	Transcriptional activation	Xu et al. (2005)
	K4 (*A. nidulans*)	Methylation	CclA	Transcriptional activation	Bok et al. (2009)
	K9 (*N. crassa*)	Methylation	Dim-5 (*N. crassa*)	Transcriptional repression	Tamaru and Selker (2001)
	K27 (*F. graminearum*)	Methylation	KMT6	Transcriptional repression	Connolly et al. (2013)
	K36 (*N. crassa*)	Methylation	Set2	Transcription activation	Adhvaryu et al. (2005)
	K79 (*S. cerevisiae*)	Methylation	Dot1	Transcriptional activation	Krogan et al. (2003)
H4	K5 (*S. cerevisiae*)	Acetylation	Esal	Transcriptional activation	Clarke et al. (1999)
	K8 (*S. cerevisiae*)	Acetylation	Esal	Transcriptional activation	Clarke et al. (1999)
	K12 (*S. cerevisiae*)	Acetylation	Esal	Transcriptional activation	Clarke et al. (1999)
	K16 (*S. cerevisiae*)	Acetylation	Esal	Transcriptional activation	Clarke et al. (1999)

Saccharomyces cerevisiae is only used as a reference if the respective modification has not yet been identified in a multicellular fungus

from the Piwi clade of animal Argonaute proteins. These siRNAs have not been identified in fungi as yet, and we thus refrain from their discussion in this article. Long ncRNAs (lncRNAs) have been identified in all eukaryotic cells including fungi (Engström et al. 2006; Katayama et al. 2005; Wang et al. 2005; Donaldson and Saville 2012). Some of them are components of the ribosome (5.8S, 18S, and 26S rRNA), but a subset of lncRNAs is the so-called natural antisense transcripts (NATs) whose sequence is complementary to other RNAs. Depending on whether they arise from the same genomic region as their complementary sense transcript or from a remote locus, they are further divided into *cis*- and *trans*-NATs. Their action appears to involve formation of double-stranded RNA, transcriptional interference, and chromatin remodeling. In fungi, they were shown to be involved in the regulation of mating and meiosis, cell aging, carbon metabolism, circadian rhythm, and plant pathogenesis (reviewed by Donaldson and Saville 2012).

To exert their regulatory effect, the siRNAs associate with Argonaute family proteins and guide them to the respective RNA targets to regulate expression of diverse genes. Based on whether or not their biogenesis is dependent on Dicer, a double-stranded RNA-specific RNAse III ribonuclease, the siRNAs are termed as Dicer-dependent (ddsiRNA) and Dicer-independent (disiRNA) groups. The ddsiRNA group includes microRNAs (miRNAs) and various small interfering RNAs (siRNAs), such as exo-siRNAs, endo-siRNAs, and natsiRNAs (Ghildiyal and Zamore 2009). They are processed by Dicer-like enzymes from stem-loop RNA precursors (Ambros et al. 2003; Bartel 2004). By analyzing small RNA associated with the *Neurospora* Argonaute protein QDE-2, Lee et al. (2010) showed that milRNAs are produced by at least four different mechanisms that use a distinct combination of factors, including Dicers, QDE-2, the exonuclease QIP, and an RNAse III domain-containing protein MRPL3 (Fig. 2). In contrast, disiRNAs originate from loci producing overlapping sense and antisense transcripts and do not require the known RNAi components for their production.

Results of the analysis of mutants in the RNAi machinery components of different filamentous fungi (Kadotani et al. 2004; Nicolás et al. 2007; Alexander et al. 2008; de Haro et al. 2009) and yeasts (Hall et al. 2003; Bernstein et al. 2012) suggest roles in controlling development, but their exact molecular functions could not be revealed. In addition, protection of genome integrity appears to be another major role: transcriptome analyses for RNAi mutants in yeasts showed an increase in transposon-derived transcripts (Drinnenberg et al. 2009; Janbon et al. 2010), suggesting that the main function of this pathway is the control of genome integrity. *N. crassa* has two different silencing pathways that are mediated by siRNAs and participate in genome defense against invasive nucleic acids such as transposon and viruses: quelling (Romano and Macino 1992) and meiotic silencing by unpaired DNA (Shiu et al. 2001). In quelling, the siRNAs cause the destruction of specific mRNA molecules (Hasunuma 2009) and require the RNA-induced silencing complex (consisting of QDE-1, QDE-2, QDE-3, Dicer, and QIP; Dang et al. 2011).

Natural antisense transcripts (NATs) are a class of endogenous coding or ncRNAs, longer than 200 nts, that have sequence complementary to other RNAs in the cell. *cis*-NATs

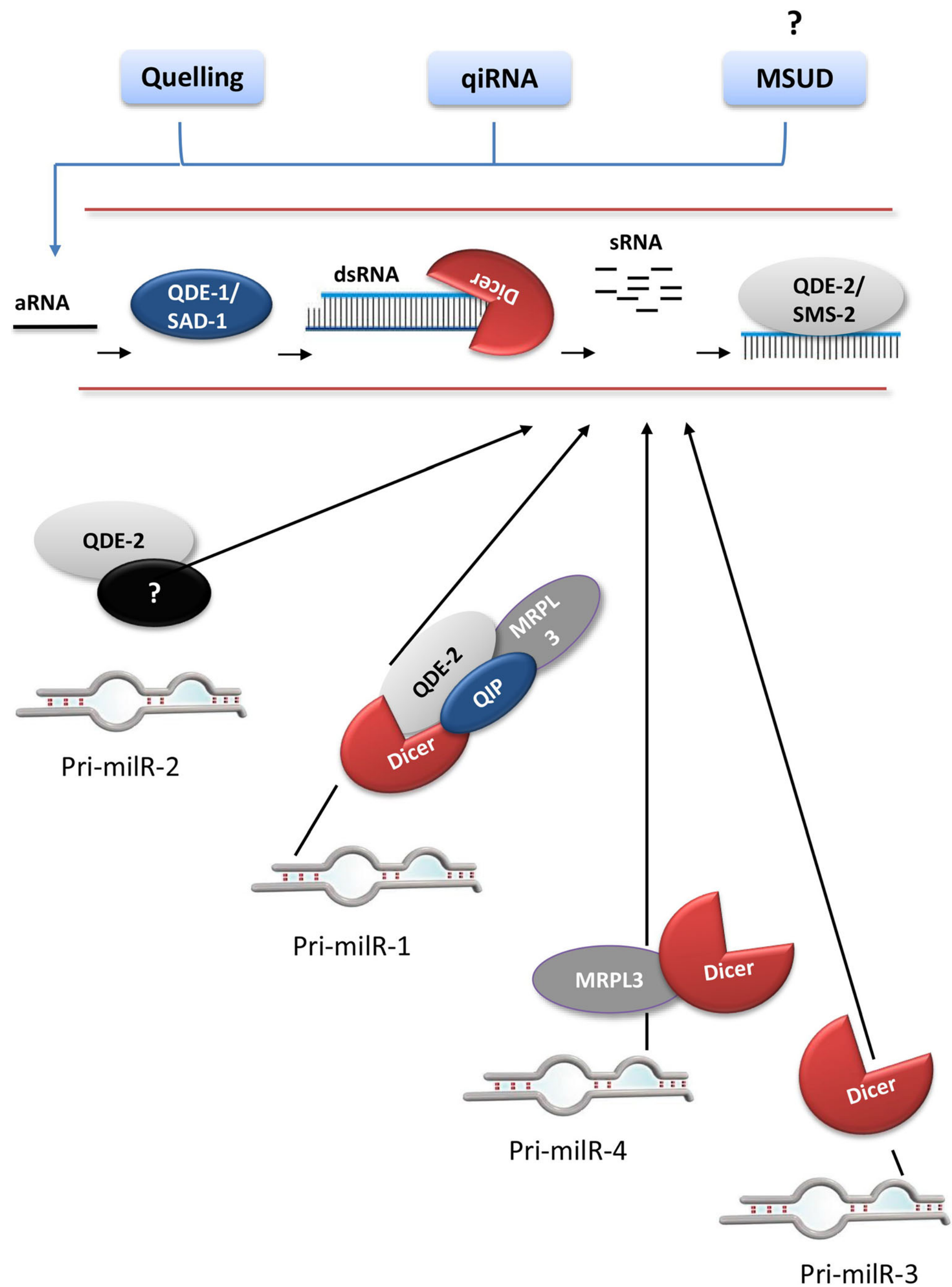


Fig. 2 Biosynthesis of microRNAs in fungi (Dang et al. 2011). *milRNAs* microRNA-like RNAs, *pri-milR* primary (transcripts) milRNA, *aRNA* aberrant RNA, *sRNA* small RNA, *qiRNA* QDE-2-interacting RNA, *MSUD* meiotic silencing by unpaired DNA, *QDE-2* quelling-deficient 2, *SAD-1* suppressor of ascus dominance 1, *SMS-2* suppressor of meiotic silencing 2, *QIP* QDE-2-interacting protein, *MRPL3* mitochondrial ribosomal protein L3. Diverse biogenesis mechanisms identified in *N. crassa* to produce eukaryotic sRNA (*black arrows*). These pathways combine different RNAi components, including Dicer, QDE-2, QIP, a putative RNase III domain-containing protein, MRPL3, and other unidentified nucleases, to produce different milRNAs. Quelling pathway and qiRNA are involved in the production of aRNA (*blue lines*); role of MSUD in aRNA production is unknown

are transcribed from the opposite strands of the same genomic locus and usually have a long perfect complementary overlap between the sense and antisense transcripts. In contrast, *trans*-NATs are transcribed from different genomic loci and often have only short and imperfect complementary. There has been much debate about whether these lncRNAs—which number in the hundreds in many eukaryotic genomes—are merely transcriptional noise with no function (Ramaiah et al. 2012). While this may be the case for some lncRNAs, recent studies have shown that some specific lncRNAs influence key biological functions in eukaryotes, including pluripotency, cell cycle, and innate immunity (Wang and Chang 2011; Kanduri 2011). LncRNAs have also been shown to modulate a wide range of molecular processes, including retrotransposon silencing, gene dosage compensation, gene imprinting, telomere length, and regulation of mRNA decay (Ramaiah et al. 2012).

Except for documenting their presence in various fungal species, the mechanisms of formation and function of the lncRNAs have not been investigated in filamentous fungi. One of the few exceptions is *qrf* of *Neurospora* (Kramer et al. 2003; Belden et al. 2011), which is an lncRNA antisense

transcript to the circadian clock gene, *frequency* (*frq;* Aronson et al. 1994). *qrf* expression affects the clock's response to light (Kramer et al. 2003) via chromatin modification at the *frq* promoter (Belden et al. 2011). A bioinformatic investigation predicted the presence of 87 pairs of sense/antisense ORFs in *N. crassa* (Steigele and Nieselt 2005). In a genome-wide study, Arthanari et al. (2014) found 939 lncRNAs, of which 477 were antisense to annotated genes. Across the whole dataset, a considerable extent of complementary overlap between the protein-coding sense and antisense lncRNAs—371 sense/antisense pairs overlapped by more than 500 nts and 236 overlapped by more than 1 kb—was detected.

Work in *Saccharomyces cerevisiae* has provided some further interesting details to this: most known yeast lncRNAs are capped, and their half-life depends on the decapping enzyme Dcp2 as proven by the increased lncRNA stability in *DCP2*-deficient yeast strains (Ramaiah et al. 2012). Intriguingly, several of these *DCP2*-sensitive lncRNAs mapped in an antisense orientation proximal to genes, which were tightly regulated by environmental cues such as the availability of iron, inorganic phosphate, or D-galactose. For the latter case, Geisler et al. (2012) identified lncRNAs overlapping with the *GAL* structural genes as well as the *GAL4* master regulatory gene and found that the *GAL* lncRNAs cause their transcriptional repression. They also found that the *GAL1* promoter is hypoacetylated in *DCP2*-deficient cells and that the addition of D-galactose to the yeast cells led to increased histone acetylation at this locus, thereby correlating with a decrease in *GAL10* lncRNA levels. GAL10-ncRNA transcription was also shown to recruit the methyltransferase Set2 and histone deacetylation activities in *cis*, leading to stable changes in chromatin structure (Houseley et al. 2008). Interestingly, the turnover of *GAL* lncRNAs depends on a noncanonical RNA decay pathway which was independent of the decapping activators Dhh1 and Lsm1 (Geisler et al. 2012).

Epigenetic regulation in fungal biotechnology

The importance of fungi as industrial producers of, e.g., primary and secondary metabolites or enzymes, has raised a great interest in the regulation of the formation of these products and how this regulation could be modified toward increasing the production. The now emerging knowledge about how epigenetic mechanisms influence regulatory processes consequently initiated investigations about the interplay of epigenetics and product formation by industrially used fungi.

Secondary metabolite production

In view of the broadly documented operation of epigenetic mechanisms in the regulation of secondary metabolite formation, industrially produced secondary metabolites, such as penicillin or cephalosporin, were among the first biotechnological products where attempts were made to improve the respective processes by epigenetic manipulation of the producing organisms.

The filamentous fungus *Penicillium chrysogenum* is the major industrial producer of the β-lactam antibiotic penicillin. Biosynthesis of this antibiotic is encoded by three genes (*pcbAB*, *pcbC*, *penDE*) which are clustered in the genome (van den Berg et al. 2008). In *Aspergillus nidulans*, the penicillin cluster is located 30 kb from the telomere of chromosome VI and bordered by DNA repeats and transposon-like elements, and these elements regulate penicillin biosynthesis through interactions with putative chromatin remodeling complexes including the LaeA-Velvet or the HDAC complexes (Shaaban et al. 2010). Deletion of a 3.7-kb distal region consisting of the putative transposable element DNA-2 in *Aspergillus nidulans* resulted in a decrease in penicillin gene expression and product formation. The genome of *P. chrysogenum* also contains a whole array of transposon-like elements, and similar elements like those described in *Aspergillus nidulans* (*vide supra*) are bordering the 56-kb region that harbors the three biosynthetic genes (van den Berg et al. 2008; van den Berg 2011). Interestingly, the higher producer strains of *P. chrysogenum* possess amplified copies of this cluster, which are always flanked by TTTACA repeats. These repeats also occur in *Aspergillus nidulans*, but amplification of the cluster was never observed (van den Berg 2011). It will be intriguing to learn if transposons have an active role in these events in *P. chrysogenum*.

Because of these findings, the LaeA-Velvet complex became a major focus for a potential improvement of penicillin production via its manipulation. Knocking down of *pclaeA*, the ortholog of *Aspergillus nidulans laeA*, resulted in drastically reduced levels of penicillin gene expression and antibiotic production, while its overexpression gave rise to a 25 % increase in penicillin production (Kosalková et al. 2009). Hoff et al. (2010) confirmed these data by *laeA* knockout strains and showed that knockout of the *velvet* A ortholog PcvelA similarly decreases penicillin production. Unfortunately, they did not report the effect of potential overexpression of these two genes. In this context, it is important to note that Veiga et al. (2012) showed that the cultivation of a high-producing strain of *P. chrysogenum* in glucose-limited chemostat condition at a growth rate typical for penicillin production (0.03 h^{-1}) revealed no influence of the velvet-LaeA complex. More recently, PcVelC—a *P. chrysogenum* ortholog of VelC—was shown to be also necessary to activate penicillin biosynthesis (Kopke et al. 2013).

An interesting additional aspect about how LaeA regulates penicillin biosynthesis in *P. chrysogenum*, which has not been found yet in *Aspergillus nidulans*, comes from the findings that its biosynthesis is triggered by the autoinducer molecule 1,3-diaminopropane (1,3-DAP) (Martín et al. 2011). This

component is part of the structure of the triamine spermidine, and indeed, spermidine—but not other polyamines—also stimulates penicillin biosynthesis. This stimulation is reflected in an increased transcription of the penicillin biosynthetic genes *pcbAB*, *pcbC*, and *penDE* (Martín et al. 2011). Interestingly, the addition of 1,3-DAP also reverted the dramatic decrease in penicillin biosynthesis in the *laeA*-knockdown mutant. Since knocking down of *laeA* in this mutant was achieved by introducing a full-length antisense *laeA* gene controlled by a heterologous glycolytic promoter (Kosalková et al. 2009) and the addition of 1,3-DAP resulted in the formation of a clear *laeA* transcript, 1,3-DAP must act at the level of siRNA regulation. In addition, since 1,3-DAP also stimulates *laeA* expression in the wild-type strain, it is possible that the RNAi generally regulates *laeA* expression in *P. chrysogenum*. The occurrence of RNAi in this species, and its dependence on Dicer, has been documented by Ullán et al. (2008) and Janus et al. (2009). Martín et al. (2011) speculated that 1,3-DAP and spermidine may enhance the binding of the RNA polymerase to the *laeA* gene promoter or enhance the stability of the *laeA* transcript maintaining high levels of the *laeA* mRNA during prolonged periods of time.

Cephalosporins are another prominent class of β-lactam antibiotics produced by fungi (e.g., *Acremonium chrysogenum*) and also bacteria (e.g., *Streptomyces clavuligerus*) (Brakhage et al. 2009). The early part of the biosynthetic pathway leading to the formation of cephalosporin in *Acremonium chrysogenum* is similar to that leading to penicillin. To investigate regulation of the cephalosporin biosynthetic pathway by the velvet complex, Dreyer et al. (2007) identified the *veA* ortholog *acveA* from *Acremonium chrysogenum* and showed that the cephalosporin C titer of knockout strains is reduced by 80 %. Interestingly, a loss of function of *acveA* also affected hyphal fragmentation, a critical parameter in the cephalosporin fermentation. No data on the effect of *acveA* overexpression were provided.

Other secondary metabolites produced by industry and shown to be regulated by the velvet-LaeA complex include compactin (ML-236B), a potent inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, which is produced by *Penicillium citrinum* (Chakravarti and Sahai 2004). It is used for biocatalytic transformation to pravastatin, a drug used for the treatment of hypercholesterolemia (Chakravarti and Sahai 2004). Baba et al. (2012) demonstrated that the genes encoding the *P. citrinum* orthologs of VeA and LaeA in *P. citrinum* control the expression of *mlcR*, the pathway-specific activator gene for compactin biosynthesis.

Monascus spp. are used to produce Monascus-fermented rice (MFR), which is frequently cited as red mold rice or Ang-Kak, and used for thousands of years in East Asian countries to produce fermented foods such as red rice, wine, and fermented soybean curd (Kuba-Miyara and Yasuda 2012). MFR is also used as a folk medicine to improve blood circulation and spleen and stomach health, and most of the health-promoting compounds have been identified to be fungal secondary metabolites. Lee et al. (2013) therefore overexpressed the *Monascus pilosus laeA* ortholog under the control of the strong *Aspergillus nidulans alcA* promoter. The resulting *OE:laeA* transformant produced four times more secondary metabolites such monacolin K, a cholesterol-lowering agent, and also components not detected under the same conditions in the parent strain. In addition, production of pigments and antioxidant activity was remarkably increased.

Kojic acid (5-hydroxy-2-(hydroxymethyl)-4-pyrone), a chelation agent produced by several fungal species but best known from *Aspergillus oryzae*, has a variety of applications in the food and cosmetics industries (Yamada et al. 2014). The expression of the genes encoding the enzymes for kojic acid biosynthesis and its production were both impaired in *Aspergillus oryzae laeA* knockout mutants disrupted in *laeA*, indicating that it is under the control of LaeA (Oda et al. 2011).

Production of extracellular enzymes

The cost-efficient application of second-generation biofuel production still depends on an improvement in the yield, efficacy, and speed of several of the involved steps, particularly biomass pretreatment, enzymatic hydrolysis (production, activity, and composition of the enzymes used), and fermentation of the pentoses in the hydrolysate (Margeot et al. 2009; Xu et al. 2009). Cellulases play a very important role in the cellulose digestion process, and so far, the cost of cellulases is very expensive due to the large amounts required for hydrolysis (Sun and Cheng 2002). Therefore, the improvement of microbial strains for the overproduction of cellulases is a major focus for biomass hydrolysis. Progress in this area has been the subject of a recent review (Dashtban et al. 2009).

Genome sequencing of the major industrial cellulase-producing fungus, *Trichoderma reesei*, has revealed that the genes encoding the various cellulases, hemicellulases, and auxiliary activities are clustered together with secondary metabolite gene clusters (Martinez et al. 2008; Kubicek et al. 2011). Marie-Nelly et al. (2014) have recently reported that several of these clusters occur at the chromosome ends. Also, several glycoside hydrolase genes are fourfold overrepresented in telomeric regions in *N. crassa* (Wu et al. 2009), thus raising the hypothesis that they may be regulated by telomere position-dependent repression.

Today, a growing body of evidences is suggesting that the regulation of cellulolytic enzymes can be performed at epigenetic levels. The first study tackling this question was the functional analysis of the *Trichoderma reesei laeA* ortholog *lae1* (Seiboth et al. 2012). Gene deletion of *lae1* indeed fully abolished cellulase gene expression, whereas *lae1* overexpression strongly increased it. However, ChIP with H3K4me2, H3K4me3, and H3K9me3 antibodies, while revealing the

expected patterns in most transcribed genes, failed to demonstrate enrichment with H3K4 or H3K9 methylation in the absence or presence of functional *lae1*: in fact, only one CAZyme gene (*cel5B*) showed enrichment of H3K4 methylation in wild type and OE*lae1*, with concomitant reduction in *Δlae1* (Seiboth et al. 2012). This indicates that, as outlined above, a direct involvement of LAE1 in histone methylation does not occur. However, its action within the Velvet complex is supported by the findings that the *Trichoderma reesei* VeA ortholog VEL1 is also essential for cellulase gene expression (Karimi Aghcheh et al. 2014). An involvement of H3K4 methylation in cellulose formation was however demonstrated in *Magnaporthe oryzae*, where a knockout of the *MoSET1* gene, encoding the methyltransferase that catalyzes H3K4 methylation (MoSET1), strongly reduced the induction of the cellulase MoCel7C by carboxymethyl cellulose (Van Vu et al. 2013). Evidence that chromatin modification is necessary for cellulase gene expression was further obtained by analyzing the nucleosome rearrangement in the promoters of the genes encoding the two major cellulases CEL6A and CEL7A of *Trichoderma reesei* (Zeilinger et al. 2003; Ries et al. 2013): under cellulase-inducing conditions, the positioning of nucleosomes downstream of the motif binding the transcriptional activator was lost and thus made the TATA box accessible for the RNA polymerase II. Recently, the *Trichoderma reesei* ortholog of the *Saccharomyces cerevisiae* HAT Gcn5 (GCN5; Trire2:64680) was shown to be essential for cellulase gene expression (Xin et al. 2013). In the absence of GCN5 function, cellulase gene expression and acetylation of H3K9 and H3K14 in the *cbh1* promoter were dramatically decreased (Xin et al. 2013). Interestingly, overexpression of yet another GCN5-*N*-acetyltransferase from *Trichoderma reesei* (Trire2:120120) causes a twofold enhancement of cellulase formation (Häkkinen et al. 2014), and its expression is downregulated in a *Trichoderma reesei Δlae1* strain (Karimi Aghcheh et al. 2013). A recent investigation of LAE1-targets in *Trichoderma reesei* cultivated at constant growth rates identified several more GCN5-*N*-acetyltransferases that are affected by loss of function of *lae1* (Fekete et al. 2014).

The control of production of extracellular enzymes by the velvet complex does not seem to be restricted to *Trichoderma*: in *Aspergillus flavus*, production of amylase and protease is also dependent on the function of *veA* (Duran et al. 2014). Similarly, the production of carbohydrate-active enzymes and proteases depends on functional *veA* and *laeA* in *Botrytis cinerea* (Schumacher et al. 2015).

The formation of carbohydrate-active enzymes by fungi seems also to be controlled at the level of RNA interference. Transcriptomic studies in *Aspergillus niger* (Delmas et al. 2012) and *Trichoderma reesei* (Ries et al. 2013) revealed that between 1.5 and 3 % of reads were antisense reads. In 630 and 521 genes of *Trichoderma reesei* and *Aspergillus niger*, respectively, antisense reads outnumbered those of sense reads (>1) in at least one condition (growth on glucose or wheat straw, respectively). Interestingly, in *Aspergillus niger*, the antisense coverage level on glucose extends over the full length of the predicted gene including the two introns and extends both upstream and downstream, whereas the sense coverage during growth on wheat straw is shorter in length, and there is almost zero coverage of the introns, indicating that the vast majority of sense transcripts are fully spliced. Among the genes of *Aspergillus niger* where sense transcription dominated on straw but antisense predominates on glucose were several permeases, carbohydrate-active enzymes, and a putative lipase (Delmas et al. 2012). This suggests that RNAi has a regulatory influence on cellulase and hemicellulase formation in *Aspergillus niger* and probably also in *Trichoderma reesei*. Interestingly, in an *Aspergillus niger* strain that bears a deletion in the gene encoding the carbon catabolite repressor CreA, the transcription of a lipase that is strongly regulated by antisense transcription is lost, suggesting that CreA is involved in the regulation of the sense/antisense switch (Delmas et al. 2012).

Concluding remarks

Although the application of epigenetic principles and mechanisms has, so far, been used for fungal strain improvement in only a small number of cases, the results have nevertheless shown that this is an area of research with high potential. It now becomes clear that DNA methylation is likely not an appropriate level for strain improvement, because its role in fungi appears to be in sexual development and defense against invading elements only. However, research on chromatin modification has already yielded a number of targets that can be used for potential strain improvement. In this regard, it is intriguing to note that most of the respective work has, so far, been performed with the enigmatic protein LaeA/LAE1 and the Velvet complex, but no research or patent has, so far, been published on the use of any of the histone methyltransferases or acetyltransferases or other chromatin-modifying enzymes. We expect that this will be an upcoming area of research in the next future. Finally, the possible role of RNA interference has just only been recorded, but attempts toward its understanding have just only begun. Work on the yeast *GAL* regulon has (as described above), however, already identified genes whose manipulation would be expected to be useful for fungal strain improvement toward enzyme and secondary metabolite production.

Compliance with ethical standards

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