Coordination of secondary metabolism and development in fungi: the velvet family of regulatory proteins

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Abstract
Filamentous fungi produce a number of small bioactive molecules as part of their secondary metabolism ranging from benign antibiotics such as penicillin to threatening mycotoxins such as aflatoxin. Secondary metabolism can be linked to fungal developmental programs in response to various abiotic or biotic external triggers. The velvet family of regulatory proteins plays a key role in coordinating secondary metabolism and differentiation processes such as asexual or sexual sporulation and sclerotia or fruiting body formation. The velvet family shares a protein domain that is present in most parts of the fungal kingdom from chytrids to basidiomycetes. Most of the current knowledge derives from the model Aspergillus nidulans where VeA, the founding member of the protein family, was discovered almost half a century ago. Different members of the velvet protein family interact with each other and the nonvelvet protein LaeA, primarily in the nucleus. LaeA is a methyltransferase-domain protein that functions as a regulator of secondary metabolism and development. A comprehensive picture of the molecular interplay between the velvet domain protein family, LaeA and other nuclear regulatory proteins in response to various signal transduction pathway starts to emerge from a jigsaw puzzle of several recent studies.

Introduction
Fungi represent one of the largest groups of eukaryotic organisms on earth, with a conservative estimate of 1.5 million mostly unknown species (Hawksworth & Rossman, 1997). They are metabolically versatile and serve as biofactories for the industrial production of various proteins and metabolites. The fungi have the potential to synthesize numerous secondary metabolites, including antibiotics such as penicillin, which was discovered almost a century ago (Fleming, 1929), but also very potent toxins such as the carcinogenic aflatoxin (Georgianna & Payne, 2009). Secondary metabolites are small, low-molecular-weight bioactive natural products that are not only produced by numerous members of the fungal kingdom, but also by plants or bacteria. Many genes for the synthesis of secondary metabolites are arranged in gene clusters, which are coordinately regulated by cluster-specific transcription factors (Brakhage & Schroockh, 2010; Evans et al., 2010). Fungal secondary metabolites include nonribosomal peptides such as the penicillins, polyketides such as aflatoxin, hybrids of these two classes as well as terpenes, indole alkaloids or oxylipins (Keller et al., 2005; Hoffmeister & Keller, 2007; Christensen & Kolomiets, 2010).

Fungal spores are dispersed in the air or by water and can therefore easily reach all parts of this planet, including potential hosts as our crops. Molds spoil approximately 10% of the world’s annual harvest and farmers in developing countries often are not aware of the threat of mycotoxins (Normile, 2010). Recent studies suggest that the world will need 70–100% more food to feed approximately 9 billion people by the middle of this century. Most likely, more food will need to be produced from the same amount of land due to competition for land from other human activities (Godfray et al., 2010). Hence, a better control of fungal mycotoxin production is required.

The control of secondary metabolism in fungi is often coordinated to fungal growth and development (Calvo et al., 2002; Yu & Keller, 2005; Braus et al., 2010). The evolutionary success of fungi is based on their ability to explore and conquer new ecological niches. A versatile aerobic heterotrophic primary and secondary metabolism is one
prerequisite combined with the specific fungal growth mode, which are normally multicellular hyphae (Fig. 1a). The hyphae are the tube-like structures that are produced after the germination of a fungal spore by the repetition of elongated cellular units. True hyphae are the basic growth units of most fungi and expand at the apex of the tip cell. Polar tip growth includes expansion of the plasma membrane and biosynthesis of cell wall components and requires the apical body of the *Spitzenkörper* as the vesicle supply center (Steinberg, 2007; Harris, 2009). Filamentous fungi are able to adhere to and invade substrates and the size of the organism is not predetermined as in a unitary organism like a human being. Therefore, an undisturbed environment as the soil of an old forest allows the formation of giant filamentous organisms that are assumed to be among the largest and oldest individuals on earth (Smith *et al.*, 1992). Hyphae of higher fungi such as the ascomycetes or the basidiomycetes usually possess septae as cross-walls at rather regular intervals. The septal units are interconnected via pores through which the cytoplasm and, in case of ascomycetes, also entire nuclei can migrate towards the growing tip as a part of a sophisticated transport and communication system of continuous interconnected cellular units (Steinberg, 2007).

Yeasts as single-cell fungi represent only a small proportion of the fungal kingdom and have been evolved in different taxonomic groups (Fig. 1a). Some of them can undergo a transition from the round single-cell yeast form to a filamentous growth mode. The yeast form reflects primarily growth on surfaces or in liquid. Fungal dimorphism between the yeast and the filamentous growth form depends on environmental conditions and can represent...
different phases of a fungal life cycle (Gimeno et al., 1992; Mösch, 2000; Klein & Tebbets, 2007; Morrow & Fraser, 2009). True hyphae can be formed by dimorphic fungi as the human pathogen Candida albicans or the plant pathogen Ustilago maydis. There are also pseudohyphae consisting of elongated cells that adhere to each other after cell division is completed without forming interconnected cellular units. Pseudohyphae are typical for diploid budding yeasts of Saccharomyces cerevisiae, but can be also formed by C. albicans (Fig. 1a).

Filamentous fungi, with the exception of the dimorphic fungi, have to develop vegetative hyphae before they can proceed to other developmental programs. Therefore, vegetative hyphae have to reach a competence state, which defines the differentiation capability of a fungal hypha under environmental signals (Axelrod et al., 1973). The competence time is dependent on the growth rate of a given environmental signals (Axelrod et al., 1973). The competence time is dependent on the growth rate of a given environment (Braus et al., 2002, 2010; Bayram et al., 2010; Rodriguez-Romero et al., 2010). Filamentous fungal development programs include the transition to asexual sporulation and to sexual fruiting bodies (Fig. 1a). This corresponds to a drastic change in lifestyle from modular filamentous to unitary fruiting bodies and reconstruction requires a functional protein degradation and assembly machinery (Braus et al., 2010; Helmstaedt et al., 2011). Fruiting bodies comprise highly specialized cells for tissue formation and asexual sporulation development (Pöggeler et al., 2006). Abolishment of the potential to form the sexual fruiting bodies of A. nidulans, which are closed cleistothecia, coincides with the loss of production of the aflatoxin precursor sterigmatocystin (Kato et al., 2003; Bayram et al., 2008b).

Asexual spores are conidia, which are single cells, comprising one or several nuclei and can be directly formed from hyphae or indirectly by phialides as spore-forming cells. Phialides can be parts of conidiophores that facilitate the dispersal of the conidia. Conidiophore formation is often initiated in competence hyphae in response to environmental signals starting with an extrusion of a specialized cell from vegetative hyphae (Adams et al., 1998; Etxebeste et al., 2010; Rodriguez-Romero et al., 2010). The numbers of cells required for asexual spore formation vary considerably. Neurospora crassa transforms vegetative hyphae into conidiophores. Aspergillus species insert additional steps, producing first foot cells from vegetative hyphae, which then grow and lead to a stalk. This ends up with a swollen head called a vesicle. The vesicle produces in a budding-like process short finger-like metulae as a first layer. The phialides form a second layer that generates by mitotic cell divisions the asexual conidia in a process, which is reminiscent of pseudohyphae formation of S. cerevisiae (Fig. 1b). The AbaA transcription factor for A. nidulans conidia formation and Tec1 for yeast pseudohyphae can be exchanged. These key transcription factors are also encoded by homolog genes, suggesting that the molecular control mechanism for both processes is similar (Andrianopoulos & Timberlake, 1994; Gavrias et al., 1996).

Asexual development is typical for most fungi, whereas the sexual reproduction cycle is less common and often not (yet) discovered. Fungi, which do not possess a known sexual cycle, had been classified as fungi imperfecti in the past. Comparative genomics suggests that many of the so-called fungi imperfecti have at least remnants of a sexual cycle genes and there are examples where this cycle could be activated (O’Gorman et al., 2009; Lee et al., 2010). Sexual sporulation is normally a more energy-consuming process than asexual sporulation in terms of the number of offspring. However, it may yield more diverse progenies as a consequence of crossing over and recombination during meiosis. Sexual development of self-sterile (heterothallic) fungi such as N. crassa requires two partners expressing compatible mating types. The hyphae of the two partners are fused by plasmogamy, followed by karyogamy that fuses the two nuclei. Consecutive meiosis and mitosis finally result in sexual progenies. Self-fertile (homothallic) fungi such as A. nidulans or the N. crassa-related Sordaria macrospora can even undergo the sexual program in the absence of a partner by fusing their own hyphae (Braus et al., 2002; Pöggeler et al., 2006; Busch & Braus, 2007).

Fruiting bodies require different specialized cells and occur in different shapes as for example the flask-shaped perithecia of N. crassa and S. macrospora or the closed cleistothecia of A. nidulans (Fig. 1a and b) (Busch & Braus, 2007). Specialized cell types serve as reproductive tissues, which are the generative asacogenous hyphae that form the ascospores. The maturing cleistothecium is surrounded by web-like sterile vegetative hyphae. Several aspergilli produce a second cell type that was originally described as chlamydospores. They are thick-walled Hülle cells whose function is to protect and nourish the maturing cleistothecium (Pantazopoulou et al., 2007; Sarikaya Bayram et al., 2010).

The outer layer of a fruiting body like a cleistothecium covers and protects the ascospores and supports their
survival under harsh conditions. A similar outer layer is present in sclerotia, where a hard outer rind tissue covers a mass of loosely interwoven hyphae at the center. Sclerotia are highly melanized vegetative structures in plant pathogenic fungi. Most sclerotia have lost the sexual propagation ability during evolution, except for Claviceps, which has a sexual cycle. This suggests that the sexual program and the formation of the outer layer are distinct programs that can be separated and are linked during sexual fruiting body formation. Sclerotia share with cleistothecia the long-term survival function under inappropriate environmental conditions such as desiccation, temperature or climatic fluctuations (Coley-Smith & Cooke, 1971; Calvo, 2008; Aliferis & Jabaji, 2010).

The molecular mechanisms for the coordination of fungal secondary metabolism and development are the focus of this review (Bayram et al., 2008b, 2010). The ecological rationale for this connection is still elusive. An attractive hypothesis is the protection of fungal reproductive structures against competitors or predators in the soil such as earthworms, nematodes, snails, insects, bacteria or other fungi. Secondary metabolites might serve as a chemical shield of the fungus against rivals. This is supported by animal experiments, which showed that fungivorous insects prefer those fungi that cannot produce secondary metabolites (Rohlf, et al., 2007; Rohlf & Churchill, 2011).

The velvet (VeA) family of fungal regulatory proteins and the coordination of secondary metabolism and development

VeA protein of A. nidulans: the founding member of the velvet family

The velvet family of regulatory proteins is fungal specific, but highly conserved among ascomycetes and basidiomycetes (Ni & Yu, 2007). The founding member of this group is the A. nidulans velvet protein VeA, which was described four and half decades ago. Strains carrying the veA1 point mutation produced more conidia and fewer fruiting bodies than wild-type strains with an intact VeA protein (Kaefer, 1965). The finding that wild-type fungi require red light to induce conidiation, whereas veA1 mutants allow the formation of conidiation in the absence of light suggested almost three decades later that the velvet protein VeA played a crucial role in red light-induced conidiation (Mooney & Yager, 1990). In 2002, the veA gene was cloned by multicopy complementation of the A. nidulans veA1 mutant strain (Kim et al., 2002). When the veA gene was deleted, the deletion phenotype differed from the original veA1 mutation. VeAΔ strains could not produce any sexual fruiting bodies even under favorable dark conditions. Overexpression of veA led to constitutive sexual fruiting body formation irrespective of light or dark conditions. The veA1 allele carries a single point mutation in the start codon (ATG) of veA, resulting in ATT. Because of this mutation, mRNA translation starts at the second AUG, leading to an N-terminally truncated mutant protein lacking the first 37 amino acids. VeA protein localization within the cell depends on illumination. The cellular VeA subpopulation increases in light in the cytoplasm, whereas darkness results in nuclear accumulation of VeA (Stinnett et al., 2007; Bayram et al., 2008b). Localization of the truncated VeA1 mutant protein is unresponsive to light and results in a significantly larger cytoplasmic than nuclear localization of the protein. In 2003, it was shown that veAΔ mutants could not produce mycotoxins of the aflatoxin family such as sterigmatocystin or the antibiotic penicillin due to the lack of transcripts for the corresponding biosynthetic enzymes. This established a connection between fungal control of secondary metabolism and development with the velvet A (veA)-encoded protein as a key factor (Kato et al., 2003).

VeA in other fungi

VeA homologs have been primarily studied in ascomycetes. Deletion of the veA gene in other aspergilli, such as the toxin producers Aspergillus parasiticus or Aspergillus flavus, further corroborated VeA as a molecular link between secondary metabolism and development (Calvo et al., 2004; Duran et al., 2007; Amaike & Keller, 2009). veA mutants of A. flavus or A. parasiticus result in the loss of aflatoxin or aflatoxin precursor production associated with the loss of the potential to develop sclerotia (Calvo et al., 2004; Duran et al., 2007). The veA Δ mutant of the opportunistic human pathogen Aspergillus fumigatus leads to a change in the interplay between metabolism and asexual spore formation, which results in nitrogen source-dependent sporulation (Krappmann et al., 2005).

The role of VeA homologs in other ascomycetes is diverse, but always related to development and secondary metabolism. Deletion of the veA homolog FvVE1 of the heterothallic plant pathogen and mycotoxic fungus Fusarium verticilloides (Li et al., 2006) results in an increased ratio of macroconidia to microconidia with decreased cell wall integrity. These FvVE1Δ mutants are unable to produce the deleterious mycotoxins fumonisin and fusarins (Myung et al., 2009). Similarly, Fvvel1 of the plant pathogen and toxin producer Fusarium fujikuroi (Gibberella fujikuroi) acts as an activator of the mycotoxins fumonisins and fusarins, but in addition, as a repressor of the dark-colored pigment bikaverin. The development of microconidia is severely reduced in Fvvel1Δ mutants (Wiemann et al., 2010). The VeA homolog PcvelA of the industrial penicillin-producing fungus Penicillium chrysogenum plays an important role in penicillin biosynthesis and is required for conidiation (Hoff et al., 2010). Consistently, the deletion of
AceVA, the veA homolog of another biotechnologically relevant β-lactam antibiotic-producing fungus, *Acremonium chrysogenum*, results in a drastically reduced expression of the cephalosporin genes. The deletion triggers earlier fragmentation of hyphae than the wild type in fermentation media (Dreyer et al., 2007). Knockouts of the veA counterpart ve-1 of the fungal model organism *N. crassa* increase assexual sporulation and reduce carotenoid biosynthesis (Bayram et al., 2008c; Olmedo et al., 2010). *Aspergillus nidulans* veA can be exchanged by *N. crassa* ve-1. Nce-1 complements the deletion phenotype of *A. nidulans* veAA, resulting in sexual fruiting bodies in the dark, asexual conidia in the light and the appropriate secondary metabolism (Bayram et al., 2008c).

Similarly, the introduction of either *P. chrysogenum* mutant complements the corresponding developmental phenotypes (Hoff et al., 2010; Wiemann et al., 2010). [Correction added after online publication 5 September 2011: in the preceding sentence the word ‘deletion’ was changed to ‘developmental’] These results corroborate that the molecular function of VeA and presumably molecular interactions with other proteins are at least conserved among different ascomycetes.

**Other members of the VeA family**

VeA represents only one of currently four members of the velvet family. VelB (velvet like B) had been described as a light-dependent regulator of fungal development and secondary metabolism of *A. nidulans*, which interacts with VeA. Deletion of the genes for both proteins results in similar, but not in identical impairments of development and secondary metabolism (Bayram et al., 2008b). Similar to what is observed in *A. nidulans*, *Ffvel2* (velB) deletion strains of *F. fujikuroi* exhibit similar defects as *Ffvel1* (veA) mutants. Deletion of both genes also results in a substantially diminished virulence on the host plant (Wiemann et al., 2010).

*VosA* (viability of spores A), which is another interaction partner of VelB, had been identified as a high-copy repressor of asexual development in *A. nidulans* (Ni & Yu, 2007; Sarikaya Bayram et al., 2010). *VosA* is also required for trehalose accumulation in spores. *vosAΔ* mutants lose the long-term viability of spores due to the rapid depletion of cytoplasmic constituents as well as nucleic acids, which makes them vulnerable to heat and oxidative stress. There is no significant loss of secondary metabolite production in *vosA* mutants in the wild-type background with an intact VeA protein, indicating a primarily developmental function. However, the *vosAΔ veAΔ* double mutation leads to loss of ST production, underlining the involvement of *vosA* in mycotoxin production (Ni et al., 2010).

*VosA–VelB* forms a dimeric complex that is present in vegetative hyphae and during development in the dark where sexual development is initiated. *VosA–VelB* provides trehalose to support the viability of asexual as well as sexual spores. Presumably, this function is fulfilled during vegetative growth, because *VosA–VelB* is only present during development in the dark when it has a second function: it supports the repression of asexual development (Sarikaya Bayram et al., 2010). In light, when asexual development is favored, hardly any *VosA* or *VelB* is detectable. *VosA- and VelB-related proteins of the VeA family* can have different functions in the differentiation of other fungi and can also be linked to distinct environmental triggers. Whereas the VeA protein of *A. nidulans* responds to light, the counterparts of the velvet family proteins in the dimorphic fungal pathogen *Histoplasma capsulatum* respond to temperature. Ryp2 (*VosA*) and Ryp3 (*VelB*) proteins of this dimorphic human pathogen have been found in a genetic screen to be essential for the temperature-dependent transition from the saprophytic filamentous phase to the single-cell pathogenic yeast form (Webster & Sil, 2008). Wild-type *H. capsulatum* cells sense high temperature (37°C) and switch to the yeast form, whereas *ryp2* and *ryp3* mutants are insensitive to an increase in temperature and produce constitutively filaments. Similar to the functions of the *A. nidulans* *VosA–VelB* heterodimer, *ryp2* and *ryp3* mutants show reduced spore viability. This suggests a conserved role for *VosA–VelB* for the viability of the spores within the fungal kingdom.

The role of VelC, the fourth member of the family, is still obscure and might be auxiliary. The deletion of *velC* in *A. nidulans* results in a slight increase in sexual fruiting body formation (Sarikaya Bayram et al., 2010).

**Velvet domains**

The four members of the velvet family, VeA, VelB, VosA and VelC, share a common velvet domain that is conserved in the fungal kingdom (Fig. 2). This velvet domain comprises approximately 150 amino acids, where hardly any known common motifs are found. Some conserved stretches of amino acids including proline residues are present in the middle of the motif. VeA–VelB and VelB–VosA heterodimers as well as homodimers of velvet proteins have been identified (Sarikaya Bayram et al., 2010). Therefore, it is tempting to speculate that the velvet domain represents a protein–protein interaction domain. Velvet domains are found in filamentous ascomycetes and basidiomycetes (Table 1). Exceptions are single-cell yeasts such as the hemiascomycete *S. cerevisiae*. Velvet domains are only found in some yeasts of the ascomycetes including *Yarrowia lipolytica* and, as described above, the dimorphic temperature-controlled *H. capsulatum*. Velvet domains are also conserved in *Zygomycetes* or *Chytridiomycetes* (Table 1). Whereas the frog pathogen chytrid *Batraochytrium dendrobatidis* carries four genes for velvet domain proteins, another chytrid, *Spizellomyces punctatus*, possesses
even more velvet domain-encoding genes (Ruiz-Trillo et al., 2007). This suggests an ancient origin of the genes for the velvet domain protein family.

The VeA protein of *A. nidulans* comprises 573 amino acids and includes a bipartite nuclear localization signal (NLS) in the N-terminal half. Furthermore, a nuclear export signal (NES) is also located in its N-terminal part. A proline-rich PEST region, which is typical for an unstable protein, is present in the C-terminal end of the protein. The N-terminally truncated *A. nidulans* VeA1 mutant protein, which shows constitutively reduced nuclear import, supports that the N-terminus of VeA is important for nuclear entry. The NLS sequence of *A. nidulans* VeA interacts with the α-importin KapA in the yeast two-hybrid system (Stinnett et al., 2007). Moreover, KapA and VeA physically interact in the dark in vivo (Bayram et al., 2008b). The VeA N-terminus is also necessary for the interaction of VeA with VelB, which is strongly reduced for the interaction of truncated VeA1 with VelB. There is currently no information on the functionality of the NES regions of VeA. The amount of *A. nidulans* VeA protein is reduced in light in comparison with the dark, but it is currently unknown whether protein stability and especially the PEST region are part of this control.

VelB is the shortest member of the velvet family, with a size of 369 amino acids in *A. nidulans*. VelB contains neither a canonical NLS nor NES signals. Efficient nuclear import of VelB requires VeA (Bayram et al., 2008b). Interestingly, VelB is the only member of the family carrying two velvet domains located in the N- and C-terminal half, respectively. VelB homologs of other ascomycete or basidiomycete members also include two velvet domains, suggesting a common ancestor of velB before the separation of both groups during evolution. This might be the result of a genome rearrangement, which either resulted in the recombination of two ancestor velvet domain genes or the duplication of the corresponding exon within an original gene with a single velvet domain. Recombinations in the past might be the reason why both velB and veA are located on chromosome VIII and vosA and velC on chromosome VII, respectively. VelB interacts with VeA and VosA and is also able to form a homodimer (Bayram et al., 2008b; Sarikaya Bayram et al., 2010). It will be interesting to explore which of the two velvet domains is required for which function.

The *A. nidulans* VosA as the third member of the velvet family includes 430 amino acids and carries as VeA the velvet domain in the N-terminal half. VosA carries an NLS sequence that provides nuclear localization, but does not support VelB nuclear transport (Sarikaya Bayram et al., 2010). VosA has been suggested to act as a transcription factor, which is supported by a transcription activation domain present at the C-terminus (Ni & Yu, 2007). The long C-terminal velvet domain of VelC as the fourth member of this family has not been analyzed as yet.

### Interplay between the methyltransferase-domain protein LaeA and the VeA family proteins

#### LaeA and secondary metabolism

In 2004, the *laeA* (loss of aflR expression A) gene was identified genetically. The *A. nidulans* laeA mutant is unable to express the AflR transcriptional activator that controls the secondary metabolite cluster for the aflatoxin precursor sterigmatocystin. The deletion of *laeA* revealed that LaeA is not only required for sterigmatocystin and penicillin biosynthesis, but also for lovastatin used in health care to lower cholesterol to prevent cardiovascular diseases (Bok & Keller, 2004). Similarly, the *laeA* homologs of other fungi are involved in the control of secondary metabolism. It is required for penicillin biosynthesis and gibberellin production in the industrially used *P. chrysogenum* and the phytopathogenic *F. fujikuroi*, respectively (Hoff et al., 2010; Wiemann et al., 2010). [Correction added after online publication: Domain architecture of the velvet family proteins and LaeA in *Aspergillus nidulans*. SAM, S-adenosyl methionine-binding site; TAD, transcription activation domain; PEST, proline (P) glutamic acid (E) serine (S) and threonine (T) rich sequence.]
in numerous filamentous fungi, except the yeast-like fungi (Reyes-Dominguez et al., 2012). The LaeA protein represents a global regulator of secondary metabolism. The protein does not possess any velvet domain. LaeA is primarily located in the nucleus, and a classical NLS region is located at the N-terminus of the protein. LaeA is required for function (Fig. 2). LaeA controls a region of 70 kb of the sterigmatocystin cluster. Artificial introduction of additional genes in this region showed that the SAM-binding domain at the N-terminus of LaeA has key functions in development. Deletion of the laeA gene causes an impairment in conidiation in _Fusarium verticillioides_ (Hoff et al., 2010). LaeA also plays an important regulatory role in _A. nidulans_ morphology, where it is required for the light-dependent support of asexual development. This function requires the presence of an intact VeA protein. A _laeA_ mutant is unable to repress sexual development by light. This event results in reverse phenotypes for the deletions of _veA_ and _laeA_: the _veA_ mutation constitutively performs the asexual program and produces no sexual fruiting bodies when _LaeA_ is present. In contrast, the _laeA_ mutant is exclusively forms cleistothecia and asexual spores are drastically reduced when _VeA_ is present. Therefore, fungal

**Table 1. Presence and studies of the velvet family proteins and LaeA in the fungal kingdom**

<table>
<thead>
<tr>
<th>Fungal groups</th>
<th>Species</th>
<th>VeA</th>
<th>VeIB</th>
<th>VosA</th>
<th>VeIC</th>
<th>LaeA</th>
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(+ ) protein is present in the corresponding group; ( – ) protein is not found in the corresponding group.

[Correction added after online publication 5 September 2011: in column VeIC of species _Fusarium verticillioides_ and _Fusarium fujikuroi_, the ‘+’ were changed to ‘–’]

LaeA and light control

LaeA does not only control secondary metabolism, but also has key functions in development. Deletion of the _laeA_ gene causes an impairment in conidiation in _P. chrysogenum_ (Hoff et al., 2010). LaeA also plays an important regulatory role in _A. nidulans_ morphology, where it is required for the light-dependent support of asexual development. This function requires the presence of an intact VeA protein. A _laeA_ mutant is unable to repress sexual development by light. This event results in reverse phenotypes for the deletions of _veA_ and _laeA_: the _veA_ mutation constitutively performs the asexual program and produces no sexual fruiting bodies when _LaeA_ is present. In contrast, the _laeA_ mutant almost exclusively forms cleistothecia and asexual spores are drastically reduced when _VeA_ is present. Therefore, fungal
responses of veAD and laeAD are antagonistic and correspond in the wild type to permanent illumination and permanent darkness, respectively. This light control function is suppressed when the N-terminal part of VeA is lacking as in the veA1 mutant strains that are studied in many laboratories (Sarikaya Bayram et al., 2010).

The trimeric VelB–VeA–LaeA (velvet) complex

In 2008, it was found that LaeA and the two velvet family proteins VeA and VelB are part of a trimeric complex that is essential to coordinate secondary metabolism and development in darkness (Bayram et al., 2008b) (Fig. 3). Therefore, the two antagonists of light control, VeA being required for sexual development, and LaeA being required for asexual development, do interact physically. The amount of the trimeric velvet complex is reduced in the presence of light when fruiting body and sterigmatocystin production are repressed. VeA forms the light-responsive bridging factor that links VelB to LaeA. Light results in less VeA protein either due to reduced biosynthesis or decreased stability or both and an additional impaired import of VeA into the nucleus. For this reason, illumination results in a limited interaction of VeA with VelB, whereas complexes with LaeA are no more detectable. The VelB–VeA dimer is formed in the cytoplasm, because VelB requires VeA for nuclear transport and the trimeric complex is only formed in the nucleus. The nuclear trimeric complex is not evenly distributed throughout the nucleus, but is concentrated at specific nuclear locations. It will be interesting to explore whether these localization sites correspond to secondary metabolite clusters. VelB and VeA are essential for vegetative body formation, whereas LaeA is essential to form sexual Hüle cells and to support asexual spore formation in an appropriate environment such as light. LaeA is also a global regulator for secondary metabolism, which is presumably modulated in its activity by the velvet family proteins VelB and VeA during development in the dark (Bayram et al., 2008b; Sarikaya Bayram et al., 2010). A recent multicopy suppressor screen revealed the additional regulator rsmA (remediation of secondary metabolism), whose over-expression remedies the lack of sterigmatocystin in veAD and laeAD strains (Shaaban et al., 2010). However, developmental dysfunctions of the veA and laeA strains cannot be rescued by the rsmA gene overexpression. Therefore, the molecular mode of RsmA function in the velvet complex remains unclear.

VelB has a second function. VelB is not only part of the trimeric complex, but also of the VosA–VelB heterodimer. VosA–VelB represses asexual differentiation during vegetative growth in a liquid culture or during development in the absence of light. Expression of VosA–VelB in light, therefore, inhibits artificial asexual development. LaeA has the opposite function and is required in light to allow asexual development. In addition, vegetative VosA–VelB activates the genes for trehalose biogenesis for spores to protect them against various types of stress. Light represses the protein levels of VelB and VosA drastically.

![Fig. 3. Interactions between velvet family proteins and LaeA in Aspergillus nidulans. The α-importin KapA supports the entry of the VeA–VelB dimer into the nucleus in the dark. In the nucleus, the VeB–VeA dimer can interact with LaeA, forming the heterotrimeric velvet complex that regulates secondary metabolism and development. VeB can also form homodimers and is part of another heterodimer, VosA–VelB. VosA–VeB represses asexual development and is required for the viability of spores by activating trehalose biogenesis. In a submerged culture during vegetative growth, VosA–VeB represses differentiation and initiates trehalose biogenesis. Light decreases the cellular levels of VosA and VeB (red lines) and allows asexual sporulation, whereas the initiated trehalose biogenesis during vegetative growth still occurs. In the dark, VosA–VeB is present and negatively regulates asexual conidiation and still supports trehalose biosynthesis. VeA interacts with the red light receptor FphA that is associated with the blue light receptors LreA and LreB. PM, Post-translational modification.](https://academic.oup.com/femsre/article-abstract/36/1/1/534052)
during development, which correlates with increased asexual conidiation. In contrast to VeA, VosA does not seem to be able to transport VelB into the nucleus. This suggests that there might either be a competition for VelB between VeA and VosA or alternative nuclear carriers for VelB. VelB is also able to form a VelB–VelB homodimer, whose function is currently unknown (Fig. 3) (Sarikaya Bayram et al., 2010).

LaeA control of velvet family proteins

Deletion strains in the genes for LaeA and the velvet family proteins show opposing phenotypes. This corresponds on the molecular level to a LaeA-mediated control of the levels of other velvet family proteins. During light response, LaeA reduces the levels of VelB and VosA and, thus, the VosA–VelB heterodimer is hardly detectable. This releases the repression of asexual development that takes place during development when it is dark. Without LaeA, the protein levels of VelB and VosA are significantly elevated and accordingly repression of asexual development is increased. Furthermore, LaeA protects VeA against significant post-translational modifications. VeA undergoes, in the absence of LaeA, a molecular size increase of approximately 10 kDa. The nature of this modification is currently unknown (Sarikaya Bayram et al., 2010). The lack of LaeA results in hyperphosphorylation of VeA, which is known to be a phosphoprotein (Purschwitz et al., 2009). This also reflects on a molecular level that LaeA is far more than a global secondary metabolism regulator and plays a key role in light control of development in A. nidulans by controlling the amount and modification of velvet family protein.

Light regulators and the trimeric complex

The trimeric velvet complex might directly perceive the light signal to coordinate secondary metabolism and development from light sensors. VeA can physically interact in the nucleus with the red light phytochrome receptor protein FphA. FphA is further associated with the blue light receptors LreA and LreB (Purschwitz et al., 2008; Bayram et al., 2010), which are the white-collar homologs of A. nidulans. The WC-1/WC-2 complex acts as a photoreceptor complex and regulates the circadian rhythm in the fungus N. crassa (Chen et al., 2010). VeA interacts with FphA through its histidine kinase (HK) domain, but phosphorylation of VeA by FphA or LreA/LreB could not be found. Because the interaction of VeA with FphA is restricted to the nuclei of the fungal mycelia, VeA might serve as a scaffold protein recruiting additional regulators to modulate gene expression. The UV-light receptor CylA affects VeA protein levels by influencing the transcription of the veA mRNAs and is described in the next paragraph in more detail (Bayram et al., 2008a).

LaeA and VeA control the formation of specific developmental cell types (Hülle cells) with a peculiar physiology

Hülle cells and the transition from filamentous to single-cell growth

Hülle cells (or described earlier as chlamydospores) are produced by different species of the Aspergillus genus, including A. nidulans and Aspergillus heterothallicus, where they are associated with cleistothecia, whereas in species such as Aspergillus protuberus and Aspergillus ustus, Hülle cells are formed in masses, but not in contact with the cleistothecia (Muntanjola Cvetkovic & Vukic, 1972; Ellis et al., 1973; Carvalho et al., 2002). These globular cells that surround the cleistothecium vary in shape between the more elongated and the globular type in different species (Carvalho et al., 2002). An average Hülle cell is around 10–15 μm in size and possesses a thick cell wall resembling a banana under the light microscope (Fig. 4). Hülle cells have germination capability that can result in mature hyphae. These cells are multinuclear and nuclei can fuse and form a macronucleus. DNA contents per Hülle cell have been determined as being 17 times greater than the amount of DNA present in asexual conidia (Carvalho et al., 2002). Already in the 1970s, it was described that the number of Hülle cells was increased in the dark and reduced in light in different Hülle cell-forming species of Aspergillus (Muntanjola Cvetkovic & Vukic, 1972).

The formation of round Hülle cells from filamentous hyphae requires a major rearrangement of the cellular architecture, including the intracellular membrane system. The A. nidulans striatin StrA localizes in the endoplasmic reticulum (ER). Striats are scaffolding proteins that have been identified in animals and fungi. Striats could play a role in restructuring the intracellular membrane system for Hülle cell formation because overexpression of the strA gene induces the artificial production of Hülle cells in a submerged culture, whereas wild-type A. nidulans cultures growing in a submerged culture form neither Hülle cells, cleistothecia nor conidiophores. Overexpression of strA enhances the early stages of fruiting body development, but is critical for later stages, resulting only in abnormally small cleistothecia that are defective in ascosporegenesis. In addition, strA overexpression impairs another filament to single-cell transition, which is asexual spore formation during conidiation and conidium germination. The additional production of a diffusible red pigment of the overexpression strain represents another link between secondary metabolism and development (Poggeler & Kuck, 2004; Busch & Braus, 2007; Wang et al., 2010).

The ImeB (inducer of meiosis) protein kinase also acts at the transition of filamentous growth to Hülle cells, light
control and secondary metabolism. ImeB shares similarities to mitogen-activated protein (MAP) kinases and cyclin-dependent kinases and is widely conserved among eukaryotes. ImeB is a serine threonine kinase that is related to the yeast Ime2 (inducer of meiosis 2) kinase, which is unstable and can be stabilized by C-terminal truncation (Bolte et al., 2002). The deletion of yeast ime2 prevents pseudohyphal growth in the presence of unpreferred nonfermentable carbon sources (Strudwick et al., 2010), corroborating a function in the transition between filamentous and single-cell growth. Ime2 has additional functions and is also involved in meiosis. Crk1, the ImeB homolog of the plant pathogen U. maydis, is necessary for sexual mating and virulence (Garrido & Perez-Martin, 2003; Garrido et al., 2004). Neurospora crassa and Cryptococcus neoformans ImeB homologs have been shown to regulate sexual development negatively (Hutchison & Glass, 2010; Liu & Shen, 2010).

Knockout strains of imeB in A. nidulans result in artificial Hülle cell formation similar to overexpression of the striatin-encoding gene strA. imeBΔ strains form normal cleistothecia with viable ascospores, but are unable to turn off sexual fruiting body formation in light similar to a lnεΔ mutant. ImeB is also located at the interface between development and secondary metabolite formation, because ImeB is essential for the formation of the aflatoxin precursor sterigmatocystin. The lack of ImeB in combination with the phytochrome red light sensor FphA in the same cell results in a completely blind strain that is not responsive to any kind of light source. This indicates that ImeB acts as an additional and may be a parallel repressor in the light-dependent repression of cleistothecia formation (Bayram et al., 2009). It is currently unknown whether ImeB directly acts on the trimeric velvet complex or on its subunits as LaeA or specific members of the velvet protein family.

Transcriptional regulators of Hülle cells

A number of Hülle cell-controlling genes have been described in A. nidulans. Besides the kinase ImeB, they include the light sensor cryA and the transcriptional repressor rosA, all of which inhibit Hülle cell formation. In contrast to cryA and rosA, overexpression of the genes for the velvet protein veA and the transcriptional activator nsdD artificially trigger Hülle cell formation similar to overexpression of the ER scaffold protein strA.

The UV-light sensor and Hülle cell repressor CryA represents the only fungal cryptochrome of A. nidulans and is conserved from chytrids to basidiomycete fungi. The cryAΔ strain forms significantly more cleistothecia in UV or blue light than the wild type (Bayram et al., 2008a). The transcripts for the corresponding N. crassa cryptochrome CRY-1 are strongly induced upon blue light exposure. CRY-1 does not possess any DNA repair activity, but is involved in the circadian rhythm (Froehlich et al., 2010). Aspergillus nidulans CryA has a dual function because it also possesses photolyase activity, which requires a direct interaction with

Fig. 4. Control of Hülle cell formation and hormonal control of sexual fruiting body development. A colored scanning electron microscopy picture of a mature cleistothecium of Aspergillus nidulans is shown. The genes ppoA- and ppoC-encoding dioxygenases are required for sexual and asexual development, respectively. PpoA is required for the indicated oxylipins. [Correction added after online publication 5 September 2011: in the preceding sentence the text ‘psiB (hydroxyl) group at C-8, resulting in (8R)-hydroxy-(9,12)-octadecadienoic ( = linoleic) acid’ and ‘psiC (hydroxyl) group in C-8 and d-lactone ring) and PpoC for psiA (hydroxyl groups at C-5 and C-8 resulting in (5S, 8R)-dihydroxy-(9,12)-octadecadienoic acid’ has been deleted]. Sexual and asexual development are characterized by different ratios between the products of ppoA and ppoC. [Correction added after online publication 5 September 2011: in the preceding sentence ‘(8R)-hydroxy-(9)-octadecanoic ( = oleic) acid (OHOA) and psiB. This ratio shifts from 1 : 8 in dark (sexual) to 1 : 3 in light (asexual).’ was replaced with ‘the products of ppoA and ppoC’. On the right, the signaling cascade for Hülle cell formation is depicted; the details are described in the text.
DNA. It is tempting to assume that there is an additional transcriptional activity that has not yet been analyzed. Deletion of the cryA gene causes abnormal formation of Hülle cells in a submerged culture (Bayram et al., 2008a). The rosaΔ strain shows a similar derepression of sexual tissue characterized by abnormal Hülle cell production in the liquid submerged media. The zinc finger transcription factor RosA, which is related to S. macrospora Pro1 (Masloff et al., 1999; Vienken et al., 2005) and represents a transcriptional repressor of Hülle cells, is also involved in repression of development when the carbon source is depleted.

The lack of CryA results in a complex misregulation in the regulatory circuit of sexual development. As mentioned above, vea transcripts are increased in cryAΔ, which affects the trimeric velvet complex. Consistently, overexpression of the vea gene also induces the production of Hülle cells in a submerged culture (Kim et al., 2002). The mechanism by which VeA activates the transcription of sexual genes and secondary metabolism is unknown. VeA triggers expression of the nsdD gene for a developmental transcriptional activator. nsdD overexpression also results in Hülle cell formation in a liquid medium (Han et al., 2001). rosa transcripts increase simultaneously, which leads to a decline in the transcript levels of nsdD at a later stage. This represses further sexual development beyond Hülle cells in a liquid medium. Further sexual development for fruiting body formation requires the Zn(II)(2)2Cys(6) transcription factor NosA (number of sexual spores) transcriptional activator (Vienken & Fischer, 2006). NosA is another conserved protein that is related to its ortholog Pro1 from S. macrospora (Masloff et al., 1999). NosA is also a paralog of the RosA repressor and both proteins share more than 40% identical amino acids. The deletion of nosA results in small fruiting bodies of 30 μm with little Hülle cells (Vienken & Fischer, 2006). The two related factors RosA and NosA play their molecular roles at different time points. RosA plays a role in very early developmental decisions and turns off the activator gene nsdD (Bayram et al., 2008a), whereas NosA activity is required later during development for Hülle cell formation at the transition from primordia and microcleistothecia to mature cleistothecia (Vienken & Fischer, 2006).

**Physiology of Hülle cells**

Induction of Hülle cells by these regulators results in a fungal tissue with a peculiar physiology. Hülle cells develop strong phenol oxidase activity due to the accumulation of laccase type II enzyme, whereas laccase I is required for asexual spore formation (Hermann et al., 1983). Hülle cells of fungi, which produce unpigmented cleistothecia, do not possess laccase II activity, suggesting that pigmentation of cleistothecia is a Hülle cell-driven process presumably for protection. The oxidation reactions of phenolic compounds in Hülle cells lead to the formation of multiple reactive oxygen species (ROS). Hülle cells, therefore, produce increased catalase, peroxidases or superoxide dismutase activities to cope with increased ROS formation. Consistently, the A. nidulans catalase peroxidase gene cpeA is expressed at the beginning of sexual development when Hülle cells start to be formed and the CpeA protein is enriched in Hülle cells (Scherer et al., 2002). The noxA gene encoding NADPH oxidase of A. nidulans produces ROS and is essential for cleistothecia formation, but not for conidiation or hyphal growth (Lara-Ortiz et al., 2003). Expression of noxA gene is also induced at the start of sexual development and inhibitors of Nox enzymes result not only in a decrease in superoxide species, but also in an accumulation of Hülle cells and primordia as cleistothecia precursors that are unable to proceed in development. Therefore, ROS are not only toxic to cells, but also seem to represent an essential endogenous signal that is required for further development of cleistothecia.

Hülle cells are centers for a number of additional enzyme activities, which are required for the degradation of cell wall components. The mutA gene responsible for mutanase, which possesses α,1,3-glucanase activity, and a chiE (chitin synthase E) gene product is localized in Hülle cells and expressed at the onset of sexual development (Wei et al., 2001; Lee et al., 2005). Cell wall material presumably serves as an energy source for the developing fruiting body and Hülle cells help to mobilize this energy by functioning as enzyme storage units. Thus, Hülle cells provide nutrients to the developing fruiting bodies and are also required for the mechanical protection of the fruiting bodies.

**The methyltransferase-domain protein LaeA controls Hülle cell formation**

LaeA plays a special role in Hülle cell morphogenesis, in addition to its requirement for secondary metabolism and its role to allow asexual spore formation in light. In the absence of LaeA, hardly any Hülle cells are formed. Consistently, the constitutively formed cleistothecia of a laeAΔ mutant are significantly smaller and similar to the small cleistothecia of the nosΔ strain. Whereas wild-type fungi produce cleistothecia with a diameter of 200 μm, laeAΔ mutants only give rise to microcleistothecia sized between 20 and 40 μm. laeAΔ strains produce only three to five Hülle cells per fruiting body, whereas wild-type cleistothecia are surrounded by approximately 80–200 cells. The remaining Hülle cells produced by the laeA mutant strain lack mutA gene expression, suggesting that they are physiologically inactive. Therefore, LaeA is crucial for the nursing function of Hülle cells during fruiting body development. These cleistothecia cannot reach their normal dimensions due to the lack of the nursing function of Hülle cells.
The Hülle cell function of LaeA does not require an intact VeA protein and can also be fulfilled in the presence of truncated VeA1 (Sarikaya Bayram et al., 2010). The role of LaeA in Hülle cells differs from the other described regulators, except the transcriptional activator NosA, because only LaeA and NosA affect the number and activity of Hülle cells. In contrast to NosA, which acts directly on DNA, LaeA might serve an epigenetic control function as it is suggested for the control of secondary metabolite clusters. nosA transcripts are almost absent in the laeAΔ mutant strain. nosA overproduction in laeAΔ increases the size of the cleistothecia, further supporting that NosA acts downstream of LaeA. Therefore, LaeA might represent an epigenetic pathway acting in parallel to the transcriptional control CryA–VeA–RosA pathway on NosA (Fig. 4) to express Hülle-specific structural genes. It is unknown whether the specific role of LaeA in Hülle cell’s morphogenesis is also reflected in an increased secondary metabolite production of this cell type. Increased LaeA-mediated induction of secondary metabolite clusters could serve as a protection for Hülle cells and for the maturing cleistothecia against competitors in the soil.

Putative interactors of velvet proteins and LaeA in the control of sexual development and secondary metabolism

Transcription factor SteA is required for fruiting body formation, but not for Hülle cells

NsdD is required early for both Hülle cell formation and cleistothecia formation, whereas the LaeA-controlled NosA acts later at the transition between small- and normal-sized cleistothecia and leads to Hülle cell formation. SteA of A. nidulans represents another transcriptional activator that is indispensable for cleistothecia formation, but not for the formation of Hülle cells. Accordingly, a steAΔ mutant strain can only produce Hülle cells, but cannot form sexual fruiting bodies. These mutants are blocked at the early stages of cleistothecia development and cannot form heterokaryons (Vallim et al., 2000). SteA represents a homeodomain transcription factor with more than 60% amino acid identities to yeast Ste12p, the transcription factor, which represents the final target of the yeast pheromone pathway required for mating (Schwartz & Madhani, 2004).

The chromatin-associated repressor RcoA (yeast Tup1p) is required for secondary metabolite production and development

The dimers VosA–VeB and VeL–VeA as well as the trimeric VeL–VeA–LaeA complex act within the nucleus. LaeA might act epigenetically and it remains to be shown whether velvet proteins act as transcription factors. RcoA represents, besides RosA, another important transcriptional repression function involved in fungal development. The exact relationship of RcoA with LaeA or the velvet proteins is currently unknown. RcoA of A. nidulans is conserved up to human and is similar to the Tup1 protein of S. cerevisiae, which represents, together with Ssn6, a general repressor domain. The Tup1–Ssn6 heterodimer cannot interact with DNA, but binds to other transcription factors and deacetylated histones, preventing RNA polymerase II from accessing its target promoters. Tup1–Ssn6 is associated with histone deacetylases. The C-terminus of Tup1 and its homologs bear seven WD (Trp–Asp) repeats for protein–protein interactions to various regulators.

Neurospora crassa RCO-1 (Tup1) participates in the control of asexual and sexual sporulation (Yamashiro et al., 1996) and is, together with RCM-1 (Ssn6p), involved in photoadaptation (Olmedo et al., 2010). The Ssn6 encoding homolog of A. nidulans is presumably essential for growth (Garcia et al., 2008). The deletion of rcoA in A. nidulans leads to drastically reduced gene expression for the sterigmatocystin cluster transcription factor AflR and loss of production of this aflatoxin precursor. rcoA mutants also have reduced transcription of the asexual regulator brlA, resulting in reduced asexual conidiation (Hicks et al., 2001). RcoA cannot develop sexual fruiting bodies because they are self-sterile (Todd et al., 2006). Overexpression of veA in an rcoA mutant background cannot rescue the mutant phenotype of rcoA, suggesting that RcoA might act downstream of VeA in a mechanism that remains to be resolved.

Control factors at the interface between primary metabolism, secondary metabolism and development

A number of parameters have to be taken into account in coordinating secondary metabolism and development. Changing nutritional conditions can overrule the light signal input of A. nidulans. Increasing the glucose concentration is one possibility to overcome light repression of sterigmatocystin production. Cultures grown with 1% glucose and exposed to light produce less sterigmatocystin than cultures grown in the dark. Increased supply of 2% glucose results in enhanced sterigmatocystin production. Glucose abundance also affects the blue-light-sensing proteins LreA and LreB and the light-dependent subcellular localization of the VeA protein. LreA, LreB and FphA, do not only modulate sterigmatocystin, but also penicillin biosynthesis (Atoui et al., 2010). Similarly, a number of nutritional conditions, including amino acid supply, nitrogen source, trace elements like metal ions or the pH, can have an important impact on secondary metabolism and development that is connected to...
additional control proteins for development and/or secondary metabolism.

Amino acid regulator Gcn4p/CpcA/CPC-1

The formation of fruiting bodies necessitates the synthesis of numerous additional proteins to differentiate into the various necessary cell types. This requires amino acids as precursors of translation. Amino acids are also required for secondary metabolism, especially for the nonribosomal peptide synthases (NRP). Aspergillus nidulans possesses genes for 27 putative NRPs, where the corresponding secondary metabolite is mostly unknown (von Dohren, 2009). Amino acid limitation in fungi results in the induction of a genetic network that induces genes for enzymes of multiple amino acid biosynthetic pathways as well as for aminocyl-tRNA synthases. This genetic system is named cross-pathway control in filamentous fungi or general control in yeast (Braus, 1991; Braus et al., 2004; Hinnebusch, 2005). Other biosynthetic pathways such as the purine biosynthesis are also induced (Mösch et al., 1991). The key transcription factor CpcA or CPC-1 activates this network in numerous filamentous fungi and is related to yeast Gcn4 and human ATF4 (Ameri & Harris, 2008). This Gcn4 family of transcription factors carries a DNA-binding multiple activation domain besides a characteristic bZIP domain for dimerization (Drysdale et al., 1995).

The supply of amino acids controls fungal development. Saccharomyces cerevisiae Gcn4 does not only provide a metabolic, but also a developmental response. Activation of Gcn4 by amino acid limitation activates the transition from single-cell yeast growth to filamentous growth (Herzog et al., 2011). Gcn4 of the dimorphic human pathogen C. albicans is part of the switch control from the unicellular yeast to the multicellular hyphal form (Sanchez-Martinez & Perez-Martin, 2001). CpcA of the opportunistic human pathogen A. fumigatus controls amino acid supply in combination with an important role in virulence (Krappmann et al., 2004). Similarly, the Gcn4 homolog VlCpcA of the fungal plant pathogen Verticillium longisporum is induced in infected plants (Singh et al., 2010).

Amino acid limitation impairs cleistothecia formation in A. nidulans (Serlupi-Crescenzi et al., 1983; Eckert et al., 1999). Aspergillus nidulans CpcA is responsible for amino acid supply during starvation and induces an arrest in fruiting body formation when amino acids are lacking. Accordingly, overexpression of cpcA leads to small cleistothecia that only contain hyphae, but no more meiotically formed ascospores. This block can be released by adding amino acids and represents a connection between the control of primary metabolism and fungal development (Hoffmann et al., 2000, 2001). The molecular targets of CpcA that mediates the blocking fruiting body formation are currently unknown.

Nitrogen source, pH, iron supply and secondary metabolism and fungal development

Growth on a nitrate medium of A. nidulans increases the production of the aflatoxin precursor sterigmatocystin combined with an increased sexual development. In contrast, growth on ammonium represses sterigmatocystin production and simultaneously increases asexual conidiation. In other aspergilli, there is a different link between nitrogen source and secondary metabolism. Nitrate serves as an inhibitor of aflatoxin biosynthesis of A. parasiticus, whereas ammonium activates the expression of aflatoxin genes (Feng & Leonard, 1998). The transcription factor AreA, which possesses a C-terminal GATA Cys(2)Cys(2) zinc finger DNA-binding domain, is the Aspergillus homolog of S. cerevisiae Gln3 (Krappmann et al., 2004) and is highly conserved in filamentous fungi. AreA controls nitrogen source-utilizing genes. AreA homologs of F. fujikuroi regulate the gibberellin biosynthesis by activating responsible genes (Mihlan et al., 2003). The AreA protein of A. parasiticus binds to the promoters of some of the aflatoxin-regulatory genes aflR and aflJ (Cary et al., 2007). areAA mutants of A. nidulans can only grow on an ammonium or a glutamine medium and are impaired in asexual sporulation and spore germination (Kudla et al., 1990).

In addition, acidic pH is required for the production of sterigmatocystin, whereas penicillin production is induced under alkaline conditions. pH regulation, therefore, acts reciprocal to the production of these two secondary metabolites. PacC is the major regulator protein for pH sensing in A. nidulans and is conserved in the fungal kingdom. pacC mutants of pathogenic fungi often lose their virulence or have an impaired invasion rate in their respective hosts (Penalva et al., 2008). PacC also controls A. nidulans development, because pacC deletion strains exhibit impaired conidiation (Tilburn et al., 1995). PacC protein binds and activates promoters of alkaline pH-dependent genes as well as penicillin biosynthetic genes (Then Bergh & Brakhage, 1998). PacC has also been proposed to act directly as a repressor on sterigmatocystin cluster genes under alkaline pH conditions (Keller et al., 1997; Ehrlich et al., 1999). The interplay between LaeA and AreA, AflR and PacC in controlling secondary metabolism remains to be elucidated.

The tetrameric HapB–HapC–HapE–HapX complex (AnCF) is also well conserved throughout the fungal kingdom and beyond from yeast to humans. This tetrameric transcription factor complex binds to the CCAAT motif in the promoter of penicillin biosynthetic genes and is essential for the expression of penicillin (Steidl et al., 1999). Penicillin biosynthesis can also be repressed by the basic helix–loop–helix (bHLH) protein AnBN1 that is encoded by an essential gene (Caruso et al., 2002). Mutants of the hapB, hapC and hapE genes do not only impair penicillin
biosynthesis, but also asexual conidiation. The HapX component is controlled by iron availability and HapX binds to the HapB/C/E complex under iron-deficient conditions (Hortschansky et al., 2007). Therefore, there has to be a link between penicillin secondary metabolite production, iron-dependent gene expression and asexual fungal development.

**Control of protein levels during development**

VosA–VelB, VelB–VeA and the trimeric VelB–VeA–LaeA complex represent a small proportion of the considerable number of positively or negatively acting proteins controlling fungal development and secondary metabolism. Besides protein synthesis, stability control and degradation of proteins represent additional measures to control the amount of regulators during distinct steps of development. In order to proceed in development, certain proteins also including regulators might have to be degraded. Autophagosomes and vacuoles are major compartments of degradation. Nuclear factors such as the velvet domain proteins are primarily degraded by the 26S proteasome after the conjugation of several ubiquitin moieties to a lysine residue of a target protein. Cullin-based multisubunit ubiquitin ligases represent a major class of ubiquitin ligases where a specific F-box protein subunit recognizes the protein substrates to be marked for degradation (Feldman et al., 1997; Skowyra et al., 1997). F-box proteins, which often recognize only phosphorylated substrates, have to be exchanged from a cullin complex if other substrate proteins should be degraded. Hence, cullin complexes have to be assembled and subsequently disassembled. Assembled cullin complexes are activated by neddylation, which is the attachment of the ubiquitin-like Ned8 to a lysine residue of the cullin scaffold proteins (Kawakami et al., 2001; Sakata et al., 2007). Disassembly of cullin complexes requires the detachment of Ned8 by the conserved COP9 signalosome multisubunit complex (Cope & Deshaies, 2003; Busch et al., 2007). Unneddylated cullin interacts to Cand1 before another round of assembly and re-entry into the neddylation cycle for another active cullin ubiquitin ligase (Pan et al., 2004).

**COP9 signalosome, Cand1 and fungal development and secondary metabolism**

Defects in the genes controlling the fungal protein degradation machinery impair the fungal clock in *N. crassa* (He et al., 2005) or *A. nidulans* fruiting body formation (Busch et al., 2003). Defects in the gene *csnE* for the COP9 signalosome deneddylation block fungal fruiting body formation in the early primordia state. Hülle cell formation is still possible, suggesting that this kind of protein degradation control is not required for this transition from hyphae to single cells. However, the *csn* mutants are highly sensitive against oxidative stress and might be blocked at the primordia stage because they are unable to cope with the internal Nox-mediated ROS signal, which is supposedly triggering the further development from primordia to mature hyphae. In addition, *csnEΔ* mutants are strongly impaired in secondary metabolism and are blind and unable to respond to light appropriately. They do not only form primordia in the dark, but constitutively even if the fungus is illuminated (Nahlik et al., 2010). An even more pronounced phenotype characterizes the two genes required for Cand1 formation. Deletion of the genes for CandA-N and CandA-C corresponding to the N- or the C-terminal part of human Cand1 results in an even earlier developmental block. Hülle cells are still formed, but sexual development produces only nests and cannot proceed to primordia. The *candAΔ* colonies are even more pigmented than *csnEΔ* colonies, which reflects an even more misregulated secondary metabolism (Helmstaedt et al., 2011). These data suggest that the sophisticated control of assembly and disassembly of cullin ubiquitin ligases is not crucial for filamentous growth or the formation of single-cell conidia or Hülle cells, but for the transition from hyphae to unitary fruiting bodies.

**Fungal F-box proteins**

The function and the substrates of most of the fungal F-box proteins are unknown. The F-box is an approximately 50 amino acid protein motif found mostly at the N-terminus of the protein and comprises specific proline and leucine compositions required for the binding of the F-box to Skp1, another subunit of cullin complexes. The C-terminal part of the F-box protein, which binds the substrate proteins, can contain various conserved patterns such as WD (Trp, Asp repeats) or LLR (leucine-rich repeats) required for the interaction with the substrate (Schmidt et al., 2009). *Neurospora crassa* F-box proteins such as FWD1 serve in the regulation of the circadian clock and are crucial for the degradation of the clock regulator protein FRQ1 (He et al., 2005). The role of most of the approximately 70 F-box protein-encoding genes of *A. nidulans* (Draht et al., 2007) remains to be explored. The GrrA protein is one of the *A. nidulans* F-box proteins involved in development. *grrA* mutants of *A. nidulans* produce sexual cleistothecia without viable spores (Krappmann et al., 2006). Similarly, the deletion of the gene for the Grr1 homolog in the plant pathogen *Gibberella zeae* is impaired in sexual development and virulence (Han et al., 2007). GrrA corresponds to *S. cerevisiae* Grr1, which has multiple functions, including the degradation of the protein kinase Ime2, the counterpart of *A. nidulans* ImeB that is described above (Purnapatre et al., 2005). The connection between F-box proteins and the stability of velvet family proteins or LaeA has not yet been...
genes: yeast Fus3p MAP kinase. The genome of (Paoletti et al., 2006; Park et al., 2003; Kraus et al., 1999; Kawasaki et al., 2002; Garrido & Perez-Martin, 2003; Kraus et al., 2003; Reyes et al., 2006; Park et al., 2008; Valiante et al., 2009). The functional homologs of the yeasts' pheromone pathway are present and expressed in yeasts and are involved in fungal development and secondary metabolism. This requires various signal transduction pathways (Fig. 5).

**Signal transduction pathways for fungal development and secondary metabolism**

The plethora of control factors that act in concert with the velvet domain family and LaeA have to be correctly synthesized, localized, activated, and degraded in response to external and internal cues to fulfill their function in fungal development and secondary metabolism. This requires various signal transduction pathways (Fig. 5).

**SteA and the MAP kinase pheromone pathway**

The pheromone MAP kinase pathway is best studied in S. cerevisiae, where it regulates pheromone signaling of the two different sexes representing α or α cells during mating. Most mutations of genes for this pathway result in a sterile (ste) phenotype characterized by the lack of response to pheromones that are small modified peptides in yeast (Schwartz & Madhani, 2004). Components of this MAP kinase cascade are also involved in the transition from the yeast growth mode to pseudohyphal growth (Kohler et al., 2002). This MAP kinase module contains the MAP kinase Fus3 required for cell fusion (fus) that finally phosphorylates nuclear transcription factors such as Ste12, which is the yeast counterpart of A. nidulans SteA. Fus3 MAP kinase receives the signal from the MAP kinase kinase (MAPKK) Ste7 that is phosphorylated by the MAP kinase kinase kinase (MAPKK3) Ste11. The three yeast kinases are assembled on the scaffold protein Ste5. Many components of MAP kinase modules have been identified and deleted in the fungal kingdom, where they are involved in fungal development or pathogenicity (Muller et al., 2007). MpkB is the homolog to the SakA MAP kinase responsible for osmoregulation. SakA is not only activated in the response to osmotic and oxidative stress, but is also required for the repression of sexual development. However, the regulator that is controlled by the SakA MAP kinase in sexual development is unknown (Kawasaki et al., 2002). The deletion of A. nidulans mkpB (corresponding to FUS3) controlling transcription factor SteA leads to a similar phenotype as the steA deletion. mkpBΔ strains are blocked in the formation of sexual fruiting bodies without losing the potential to form Hülle cells and are impaired in asexual sporulation. Furthermore, the levels of the aflatoxin precursor sterigmatocystin are decreased in mpkB deletions due to the reduced expression of the aflR regulator gene as well as structural genes of the sterigmatocystin cluster. Similarly, genes for penicillin and for terrequinone biosynthesis are decreased in the mpkB strain. The MpkB-mediated signal transduction pathway does not affect LaeA control (Atoui et al., 2008). The deletion of A. nidulans steC (encoding the Ste11 MAPK3 counterpart) located upstream of the transcription factor SteA and the MAP kinase MpkB also affects sexual and asexual development (Wei et al., 2003). steCA mutants form Hülle cells, but cannot complete the sexual cycle and produce only initial nests that are blocked for further development. These mutants cannot form stable heterokaryons.

**Oxylipin hormones and fruiting body formation in A. nidulans**

Activation of the yeast pheromone Fus3 MAP kinase pathway requires two different sexual hormones, α-factor and γ-factor, which are 10–12 amino acid peptides produced by the different sexes and that are recognized by the pheromone receptors Ste2 or Ste3 of the opposite sex. Binding of pheromones triggers the Fus3 pheromone pathway and results in mating. Peptide pheromones are not known in aspergilli.

A different class of pheromones are the psi (precocious sexual inducer) factors that are oxylipins derived from lipid acids. psi factors represent a mixture of different hydroxyl-noleic acid moieties, which can induce sexual development and repress asexual conidiation pathway (Champe et al., 1987; Champe & el-Zayat, 1989). They are related to prostaglandin hormones secreted by various mammalian tissues. Receptors for oxylipins have not yet been identified in aspergilli. psi factors are produced by dioxygenases like the enzymes encoded by ppoA and ppoB. The proteins PpoA and PpoB are similar to mammalian prostaglandin synthetases and act as antagonists. The ratio between the formation of the three described psi factors psiA, psiB and psiC determines the balance between asexual and sexual development. PpoA is required for psiB (10-hydroxy-9,12-octadecadienoic acid) and psiC (8-hydroxy-9,12-octadecadienoic acid) biosynthesis, which are prerequisites for sexual development. psiC seems to be the most potent psi pheromone. The deletion of ppoA gene results in increased asexual development. PpoC produces psiA (8-hydroxy-9,12-octadecanoic acid), which is required for asexual sporulation (Fig. 4). The ppoB deletion increases sexual development and the same phenotype results from overexpression of ppoA (Tsitsigiannis et al., 2004a, b). The psi factors do not only regulate fungal development, but also secondary
metabolite production. ppoA/ppoC double mutants cannot produce sterigmatocystin, whereas the lack of ppoB increases sterigmatocystin levels (Tsitsiænissi et al., 2005; Yu & Keller, 2005).

The receptors, the signal transduction pathways and the transcriptional regulators that are required for psi hormone-mediated support of fungal development are currently unknown. The psi pathway is impaired if the neddylation/deneddylated-mediated control of protein stabilities is no more functional. Deletion of the gene for the fungal COP9 signalosome deneddylase alters secondary metabolism by inducing the orsellinic acid biosynthetic cluster or by accumulating intermediates of sterigmatocystin biosynthesis. In addition, defects in the COP9 signalosome also alter the overall ppoC expression levels during development and even earlier in the vegetative phase and this expression pattern is combined with an increased expression of ppoA. This results in a similar effect of increased sexual development. A ppoC deletion or overexpression of ppoA might be one of the reasons why this mutant strain is unable to repress sexual development in light (Nahlik et al., 2010). The targets of the COP9 signalosome in the psi pathway are unknown.

The protein kinase A (PKA) pathway

The PKA pathway is also well studied in yeast, where it can cross-talk to MAP kinase signal transduction pathways (Mösch et al., 1999; Lengeler et al., 2000). PKA catalytic subunits phosphorylate target proteins in the cytoplasm as well as in the nucleus. Target proteins of PKA for fungal development or secondary metabolism are currently unknown, but it is known that this pathway is important for fungal development and secondary metabolism. PKA activation requires binding of cyclic AMP, which releases the catalytic subunits from the regulatory subunits of PKA. The adenylate cyclase acts upstream of PKA and elevates cellular cAMP levels. The PKA pathway activates adenylate cyclase in response to nutritional conditions, including glucose supply as the carbon source that is provided by the environment. Glucose supply can affect fungal development as described above and the PKA pathway is also involved in fungal pathogenicity (D’Souza & Heitman, 2001; Shimizu et al., 2003; Banno et al., 2005; Ni et al., 2005; Huang et al., 2007). Aspergillus nidulans possesses two genes for catalytic subunits of PKA, pkaA is the major player acting in a manner opposite to pkaB as a minor player. pkaA/pkaB double-deletion strains are not viable. pkaA is required for vegetative growth and germination of conidia. Overexpression of pkaA results in a repression of asexual spore formation and a loss of sterigmatocystin production, suggesting a second function in supporting secondary metabolism (Ni et al., 2005).

The diacylglycerol-dependent protein kinase C (IP3-DAG-PKC or PKC) pathway

Another signaling pathway that is important for fungal development is the cell integrity or the PKC pathway (Heinisch, 2005). The key component PKC phosphorylates target regulatory proteins. PKC is activated by diacylglycerol and Ca++ ions. Phospholipase C acts upstream of PKC and can respond to damages in the cell wall and breaks phosphoinositol phosphate into inositol-3-phosphate (IP3) and diacylglycerol. The A. nidulans genome encodes the two PKC-encoding genes pkaA and pkbB, which are both essential. PkaA shows higher degrees of amino acid sequence similarities to the corresponding genes of fungi other than PkbB. pkaA antisense expression supports a link to fungal development because the formation of asexual conidia is reduced. pkbA also inhibits secondary metabolism by controlling AnBH1 penicillin regulatory protein, which represses penicillin biosynthesis. Reducing the pkbA leads to an increase in penicillin production by sequestering AnBH1 to the cytoplasmic fraction and therefore relieving the expression of penicillin biosynthetic genes (Caruso et al., 2002; Herrmann et al., 2006).

G-protein-coupled receptors (GPCRs)

Numerous signals for MAP kinase, PKA or PKC pathways are received by membrane-bound GPCR proteins (Fig. 5). A transmembrane protein consisting of seven transmembrane domains senses the external signal. Signals are received by the signal perception domains of GPCR located outside of the cytoplasmic membrane and result in a conformational change in the domain found inside the cell. This domain is in contact with the heterotrimeric G-proteins that transmit the signal further downwards elements (Lengeler et al., 2000; Yu & Keller, 2005; Yu, 2006).

The A. nidulans genome encodes 16 GPCR proteins named GprA-P and NopA, respectively (Han et al., 2004b; Lafon et al., 2006; Yu, 2010). PreB (GprA) and Pre3 (GprB) are closely related to the yeast pheromone receptors (Ste2 and Ste3) and are presumably the sensors for the A. nidulans MpkB/Fus3 MAP kinase pathway. GprA and GprB are involved in homothallism, which is the self-fertility that allows A. nidulans to produce the fruiting bodies in the absence of an additional partner (Seo et al., 2004). The gprD gene is involved in the control of sexual development. gprD mutants are delayed in growth and spore germination, and sexual development takes place irrespective of illumination (Han et al., 2004a, b). nopA encodes an opsin homolog protein similar to NOP-1 of N. crassa (Bieszke et al., 1999), but nopA mutants do not manifest any phenotype under tested conditions (Rodriguez-Romero et al., 2010).
Heterotrimeric G-proteins (αβγ) are located downstream of GPCRs. Mutants in heterotrimeric G-protein subunits have been isolated and characterized in *A. nidulans* many years ago as fluffy mutants (Wieser et al., 1997). The fluffy colonies are produced as a result of profusions of aerial hyphae. *fadA* (fluffy autolytic dominant A) encodes the major *Gα*-subunit of *A. nidulans*. *fadA* mutants do not only show a fluffy phenotype, but are also unable to produce sterigmatocystin (Yu et al., 1996; Wieser et al., 1997). The active form of FadA (FadA-GTP) supports vegetative growth and represses differentiation and mycotoxin production. *flbA* is the regulator of G-protein signaling, which increases the GTPase activity of FadA. The lack of *flbA* causes symptoms similar to those observed in *fadA* mutants, which are characterized by a fluffy phenotype and lack of sterigmatocystin biosynthesis (Yu et al., 1999; Yu & Keller, 2005). *ganA* and *ganB* represent two additional *Gα*-encoding genes (Chang et al., 2004). *ganB* is responsible for cAMP levels in response to carbon source availability during germination of spores and, therefore, important for the PKA pathway.

In addition to *Gα*-subunits, several Gβ-units have been investigated including *sfaD*. *SfaD* (suppressor of *flbA*) inhibits inappropriate sporulation (Rosen et al., 1999; Seo & Yu, 2006). *sfaD* mutants still produce Hülle cells, but sexual development is impaired. These developmental defects are associated with the loss of sterigmatocystin production. Phosducin proteins modify *Gαβγ* functions. Deletion of the phosducin-encoding gene *phnA* results in similar defects in development and sterigmatocystin production as *sfaD* mutants (Seo & Yu, 2006). A single *Gγ*-subunit encoded by the *gpgA* gene is necessary for sexual development and mycotoxin production. Therefore, heterotrimeric G-protein signaling as well as modifiers of this pathway play major roles in fungal development and secondary metabolism.

**Concluding remarks and future perspectives**

*Aspergillus nidulans* represents an amenable model system to study the fungal control of secondary metabolism and...
development. The insights gained from these studies might be important for fungi relevant to economy and human health. The velvet domain family and LaeA are central players in this coordination process and increasingly more pieces of the puzzle are being assembled. This improves our understanding of the interplay between regulators, the control of their protein synthesis, stability, localization and activity. The exact molecular connection between these regulators and the sensed signals like light, CO₂/O₂ levels, glucose, amino acids, trace elements, pH or ROS remains to be explored. There are many questions that remain to be answered. What type of signal is received by each receptor? What is the target of each specific signal? Which protein complexes are the targets of the PKA, MAP kinase or the PKC signaling pathways? Which target proteins are activated or inactivated? This includes the characterization of the real signals, their sensors and their regulation. The exact mechanism of psi hormone function is still elusive. Furthermore, the interplay between the various regulatory G-proteins has to be explored. Similarly, the cross-talk between the different signal transduction pathways has to be determined as well as the interplay between the final players that regulate transcription, chromatin and degradation of regulators.

Vea can serve as a scaffold to recruit more proteins involved in coordination of development, and this role should be explored in other less related fungal taxa. Is a connection to a nonvelvet LaeA-like protein conserved? Is the LaeA control of velvet domain protein levels and their modification conserved? What are the other interactors that mediate signals other than the FphA–LreB–LreA, which provides a light response? Are LaeA proteins of other fungi also involved in the formation of specific cells such as the Hülle cells of A. nidulans? What is the exact role of the velvet domain and what is the structure? How does LaeA activate gene clusters or heterochromatin in general? The answers to all of these questions will not only improve our understanding of fungal development and secondary metabolism, but might help to control and manipulate food-related or biotechnologically relevant fungi.

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References


The velvet family regulatory proteins of the fungal kingdom


