



Synthesis and Regulation of Fungal Secondary Metabolites

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Abstract

Fungi are well known for their ability to produce a multitude of secondary metabolites (SMs) which act as a weapon of defense to protect themselves against parasites and predators. A variety of fungal SMs have proved to serve as an important factor for decades. The synthesis of SMs in fungi is a complex, multi-step process and is stage-specific under specialized conditions. SMs are primarily synthesized by non-ribosomal peptide synthetase (NRPS) or polyketide synthase (PKS) enzymes. The genes encoded for SM synthesis are often located in the cluster form at the sub-telomere region. The SM biosynthetic gene cluster comprises of genes encoding for NRPS/PKS, a transcription factor, and other accessory genes essential for assembly and maturation of SM. The regulation of SM synthesis in fungi can be achieved by pathway-specific (in-clustered transcription factor), global regulatory proteins and chromatin remodeling. The regulatory protein-encoding gene present in each gene cluster is considered to be a crucial regulatory circuit of the SM biosynthetic pathway. Moreover, the regulation of fungal SM biosynthesis is also guided by global regulatory proteins responsive to pH, carbon, nitrogen, light/dark, and other environmental cues. Histone modifications by methylation and acetylation often altered the chromatin structure; as a result, these changes repress or express the genes of SM biosynthetic pathway. Taken together, we conclude that fungal SM ability as antibiotics to toxins is useful to mankind.

Keywords

Persistence toxic substances · Bioremediation · Biodegradation · Genetically modified microorganisms

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2.1 Introduction

The enormous variety and diversity of fungi stand a prime place in the natural world. The geographical diversity of India is the most suitable to fungi that contributes more than half of the total fungal species of the world. However, the flora of the fungal kingdom is still undiscovered; thus it needs to be explored for detection of novel therapeutics for the treatment of a plethora of pathogens. Fungi are well known for their ability to produce a multitude of secondary metabolites (SMs) as products. SMs are small organic molecules (<4 kDa) which are unlikely to be involved in cell growth and development but have a pivotal role in self-defense, niche adaptation, and communication to the environment (Kamei and Watanabe 2005; Keller et al. 2005). SMs isolated from fungi have been considered the most resourceful natural product as they have been exploited by humankind as antimicrobial, antitumor, enzyme inhibitor, immunosuppressive, anticholesterol, and anti-parasitic agents (Agathos et al. 1987; Demain 1999; Ramawat and Mérillon 2013). Despite their clinical importance, fungi also synthesize some toxic SM substances which are sometimes deleterious to human beings as they can cause systemic infections (Demain and Fang 2000; García-Estrada et al. 2011). A range of SMs with their effectiveness and producing organisms are listed in Table 2.1. Characteristically, synthesis of SMs in fungi is stage-specific as they are produced by a specific organism/group of organisms under certain growth/environmental conditions such as media, pH, temperature, and nitrogenous sources (Jensen et al. 2007; Fox and Howlett 2008; Brakhage 2013; Keller 2015). SM production most often occurs at the late log phase or stationary phase (Bu'Lock 1961). Earlier observation revealed that environmental conditions for both SM production and spore formation were found to be common, but they were more stringent than vegetative growth (Bu'Lock 1961; Sekiguchi and Gaucher 1977). Therefore, it is believed that synthesis of SMs is essential for sporulation. For example, the dark-brown melanin pigments synthesized by oxidative polymerization of phenolic compounds which are deposited in the cell wall during cell sporulation protect the spores from UV light and are also involved in the virulence (Calvo et al. 2002). Aside from melanins, zearalenone produced in *F. graminearum* also induces the sporulation (Wolf and Mirocha 1973). The fungal SM production is also linked to the asexual to sexual development. The switching from primary to secondary metabolism or asexual to sexual development depends on the psi factor or endogenous ratio of oleic acid- and linoleic acid-derived molecules (Champe and El-Zayat 1989; Calvo et al. 2001). It is well known that complex and diverse SMs derived from fungi are synthesized by sequential assembly of primary metabolic intermediate precursors molecule, i.e., acetyl coenzyme A (acetyl-CoA), 1-deoxyxylulose 5-phosphate, and mevalonic and shikimic acid (Dewick 2002). Structurally, fungal SMs are classified as non-ribosomal peptides, polyketides, indole alkaloids, and terpenes (Keller et al. 2005). The synthesis of SMs is a multi-step process which is initiated by the action of various classes of enzymes. However, a variety of tailoring enzymes, viz., transporters, oxygenases, hydroxylases, and regulatory proteins, are also involved in the biosynthesis of

Table 2.1 Fungal secondary metabolites, sources, and their medicinal importance

S. no.	Name of the species	Compound	Properties	References
1	<i>Acremonium chrysogenum</i>	Cephalosporin	Antibacterial	Sándor et al. (1998)
2	<i>Penicillium chrysogenum</i>	Penicillin	Antibacterial	Raper (1946)
3	<i>Acremonium</i> spp.	Spirobenzofuran	Antibacterial	Kleinwaechter et al. (2001)
4	<i>Zygosporium masonii</i>	Zygosporin A	Antibacterial	Hayakawa et al. (1968)
5	<i>Alternaria</i> spp.,	Altersetin	Antibacterial	Hellmig et al. (2002)
6	<i>Penicillium griseofulvum</i>	Griseofulvin	Antifungal	Grisham et al. (1973)
7	<i>Cryptosporiopsis</i> cf. <i>quercina</i>	Cryptocandin	Antifungal	Strobel et al. (1999)
8	<i>Mycogone rosea</i>	Roseoferin	Antifungal	Degenkolb et al. (2000)
9	<i>Penicillium simplicissimum</i>	Xanthoepocin	Antifungal	Igarashi et al. (2000)
10	<i>Emericella rugulosa</i>	Echinocandin B	Antifungal	Kumar et al. (2018)
11	<i>Pestalotia heterocornis</i>	Paclitaxel (Taxol)	Anticancerous	Noh et al. (1999)
	<i>Taxomyces andreanae</i>			Stierle et al. (1993)
12	<i>Pestalotiopsis microspora</i>	Torreyanic acid	Anticancerous	Noh et al. (1999)
13	<i>Streptomyces verticillus</i>	Bleomycin	Antitumor	Umezawa et al. (1966)
14	<i>Aspergillus</i> and <i>Trichoderma</i> spp.	Gliotoxin	Immunosuppressive	Pahl et al. (1996)
15	<i>Aspergillus parasiticus</i> <i>Aspergillus flavus</i>	Aflatoxins	Mycotoxin	Dorner et al. (1984)
16	<i>Penicillium viridicatum</i>	Ochratoxin A	Mycotoxin	Scott et al. (1970)
17	<i>Claviceps purpurea</i>	Ergotamines	Mycotoxin	Amici et al. (1967)
18	<i>Aspergillus</i> and <i>Penicillium</i> spp.	Patulin	Mycotoxin	Moss (2002)
19	<i>Pestalotiopsis microspora</i>	Pestacin and isopestacin	Antioxidant	Harper et al. (2003)
20	<i>Nodulisporium</i> spp.	Nodulisporic acids	Insecticidal	Ondeyka et al. (1997)
21	<i>Chaetomium globosum</i> NK10	Chaetoglobosin A	Antinematodes	Hu et al. (2013)
22	<i>Mycena galericulata</i>	Strobilurin	Antiparasitic	Hosokawa et al. (2000)
23	<i>Acremonium tubakii</i>	Cephaibol	Antiparasitic	Vertesy et al. (2003)
24	<i>Phycomyces blakesleeanus</i>	Ferritin	Parkinson's disease	Mann et al. (1994)
25	<i>Ashbya gossypii</i>	Riboflavin	Nutrient	Wei et al. (2013)
26	<i>Gibberella fujikuroi</i>	Gibberellin	Nutrient	Tudzynski (1999)
27	<i>Penicillium</i> spp.	Arisugacin	Acetylcholinesterase inhibitor	Omura et al. (1995)

fungal SMs, and their encoded genes are often arranged in a cluster (Fox and Howlett 2008; Keller 2015). The reason why SM biosynthetic genes present together as clusters is still unanswered. It is speculated that all the essential genes for an SM synthesis are transcriptionally regulated by an intrinsic transcription factor (TF) under certain environmental conditions (Fox and Howlett 2008; Keller 2015).

2.2 Different Classes of Fungal Secondary Metabolites

2.2.1 Non-ribosomal Peptides

Non-ribosomal peptides are an enormous class of SMs which are derived from both proteinogenic and non-proteinogenic amino acids. SMs belonging to this class are synthesized by the large multifunctional and multi-modular enzymes called non-ribosomal peptide synthetases (NRPS) and synthesized independently to ribosomes (Stein et al. 1996). NRPS are organized in the modules, which are highly specific to a particular amino acid and also considered as an assembly unit (Fig. 2.1a). The number of modules depends on the length of the peptides. NRPS module characteristically consists of three basic subunits named as adenylation (A), peptidyl carrier protein or thiolation (PCP or T), and condensation (C) domains (Fig. 2.1b). Adenylation domain tends to activate the amino acid to amino-acyl adenylate by reacting with ATP (Fischbach and Walsh 2006). This activated amino acid is then transferred to the PCP domain which forms a thioester bond with the cysteine-thiol group of the PCP domain (Strieker et al. 2010). However, the condensation domain (C) catalyzes the peptide bond formation between amino acid bonded to the thiolation domain and nascent peptide chain on the preceding module. In addition to the minimal core module, NRPS enzymes also contain associatory domains such as cyclization (Cy), epimerization (E), oxidation (Ox), thioesterase (TE), and methyltransferase (MT) domains essential for maturation of SM molecule (Hoffmeister and Keller 2007; Strieker et al. 2010; Evans et al. 2011). The cyclization domain (Cy) is a variant of the classical C domain which introduces thiazoline or oxazoline ring on β -heteroatom-bearing amino acids such as threonine, serine, and cysteine. The heterocyclization of participatory amino acids of SM prevents them from the action of cellular protease (Marshall et al. 2001; Kelly et al. 2005).

Initiation modules usually lacking C domain and termination modules usually possess an essential thioesterase (TE) domain at their C terminal. TE domain catalyzes product hydrolytic cleavage or complex macro-cyclization and releases the peptide molecule (Fig. 2.1b) (Strieker et al. 2010). A range of non-ribosomal peptides are methylated by MT domain which contain C-methyl or N-methyl groups containing amino acids using *S*-adenosyl methionine (SAM) as co-substrate (Walsh et al. 2001). Epimerization (E) domain is meant for the conversion of L-amino acids to the corresponding D-isomer of the peptides (Linne et al. 2001). Penicillin, cephalosporin, cyclosporine, echinocandin B, cryptocandin, and gliotoxin are the common examples of NRPS-mediated SMs (Cacho et al. 2012).

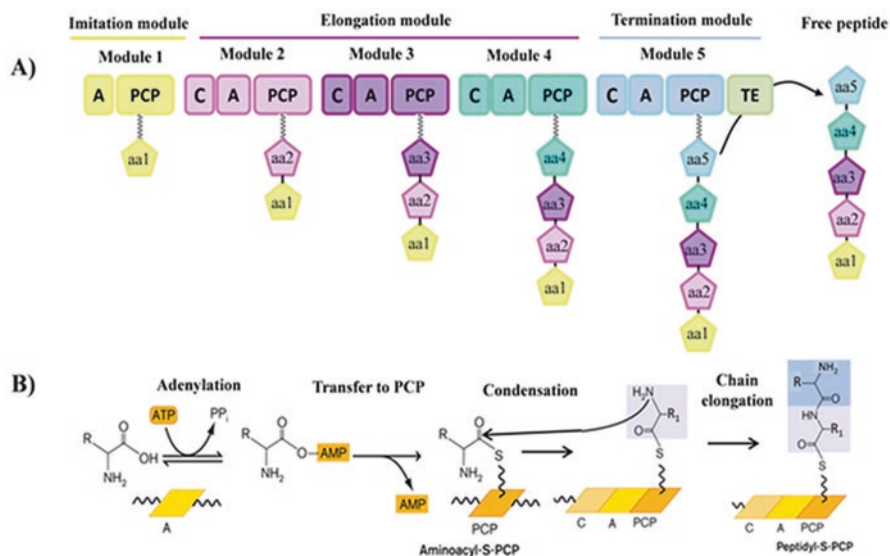


Fig. 2.1 Organization of multi-modular non-ribosomal peptide synthetase (NRPS). (a). Arrangement of different enzyme modules of NRPS. Each module contains adenylation (A), peptidyl carrier protein or thiolation (PCP or T), and condensation (C) domains. Amino acids are sequentially assembled by NRPS enzyme. Initiation module lacking C domain and termination module contains thioesterase domain (TE) which is involved in the cleavage. (b). Specified domains catalyze the amino acid to the peptides. Adenylation activates amino acid to amino-acyl adenylate by reacting with ATP, and it attaches to the PCP. Condensation domain catalyzes peptide bond formation between amino acid bonded to the thiolation domain and nascent peptide chain on preceding module

2.2.2 Polyketides

Polyketides are the structurally diverse class of fungal secondary metabolites which are primarily synthesized from the monoketide units (acetyl-CoA, methylmalonyl-CoA, and malonyl-CoA) derived from the primary metabolite pool (Keller et al. 2005; Wakimoto et al. 2011). Fungal polyketides are synthesized or assembled by polyketide synthases (PKS) which are closely related to the fatty acid synthases (FAS) (Hutchinson 2003). Similar to NRPS, PKS are also multi-modular enzymes, having ketoacyl CoA synthase (KS), acyltransferase (AT) and acyl carrier (ACP), and ketone-reducing enoyl reductase (ER), dehydratase (DH), and ketoreductase (KR) domains (Fig. 2.2) (Keller et al. 2005). PKS can be classified into three classes, PKS I, II, and III, which have identical enzymatic functions, but varying chain initiation and termination steps (Gallo et al. 2013). PKS I are complex and the most versatile in nature which can be sub-categorized into iterative and non-iterative type.

Iterative PKS I are multi-enzymes which produce non-reducing (NR), partially reducing (PR), and highly reducing (HR) polyketides. Non-iterative PKS I are organized into modules having multiple domains acting once and catalyze one cycle of polyketide chain elongation. The antibacterials orsellinic acid and erythromycin A and cholesterol-lowering agent

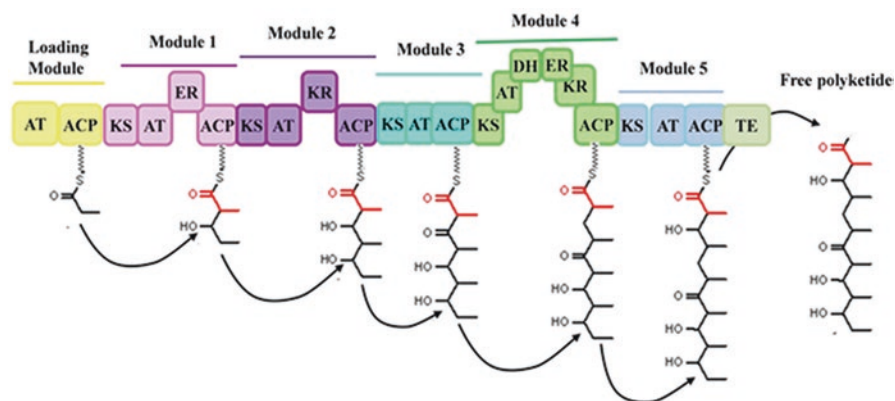


Fig. 2.2 Domain organization of the PKS. The functional domains are represented by box. Loading module lacking KS domain and terminal module has a TE domain. Each module is responsible for the specific polymerization of single monoketide unit, and the specific action of respective domains synthesizes the polyketide molecule. Domains are represented as *AT* acyltransferase, *ACP* acyl carrier protein, *KS* ketosynthase, *KR* ketoreductase, *DH* dehydratase, *ER* enoyl reductase, *TE* thioesterase, and *CoA* coenzyme A

squalestatin S1 are the known examples of iterative PKS I (Weissman 2009). PKS II are the aggregated complex of mono-functional proteins mainly comprising of ketosynthase (*KS*) α and β and acyl carrier protein (*ACP*). Each protein has a specific role in the synthesis and acts as iteratively. The iterative action of these proteins led to polyketone synthesis via chain extension to the defined number of cycles. PKS III, also known as chalcone synthases, are the homo-dimeric enzymes, which act iteratively. PKS III have simple, versatile, and broad substrate specificity and are very less abundant in fungi. They mostly utilize CoA thioesters as a substrate for decarboxylative condensation and cyclization reaction (Calne et al. 1989; Staunton and Weissman 2001; Shen 2003; Weissman 2009). The tri- and tetra-ketide pyrones, the penta- and hexa-ketide resorcylic acids, griseofulvin, bikaverin, and the penta-ketide resorcinols are such examples (Hashimoto et al. 2014; Liu et al. 2015).

2.2.3 Terpenoids

Terpenoids or terpenes are the odoriferous SMs which are mostly derived from plants (turpentine and camphor) and less commonly found in fungi (carotenoids, aristolochenes, trichothecenes, gibberellins, and indole-diterpenes). The basic unit of terpene is monomer isoprene (C_5H_8) which undergoes polymerization catalyzed by terpene synthases. The empirical formula of terpenes is $(C_5H_8)_n$, where n represents the number of isoprene units. Structurally, terpenes can be cyclic or linear and unsaturated or saturated. Two abundant building blocks of terpenoids are isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), derived from acetyl-CoA by glyceraldehyde 3-phosphate and pyruvate or mevalonic acid (Fig. 2.3) (Eisenreich et al. 2004).

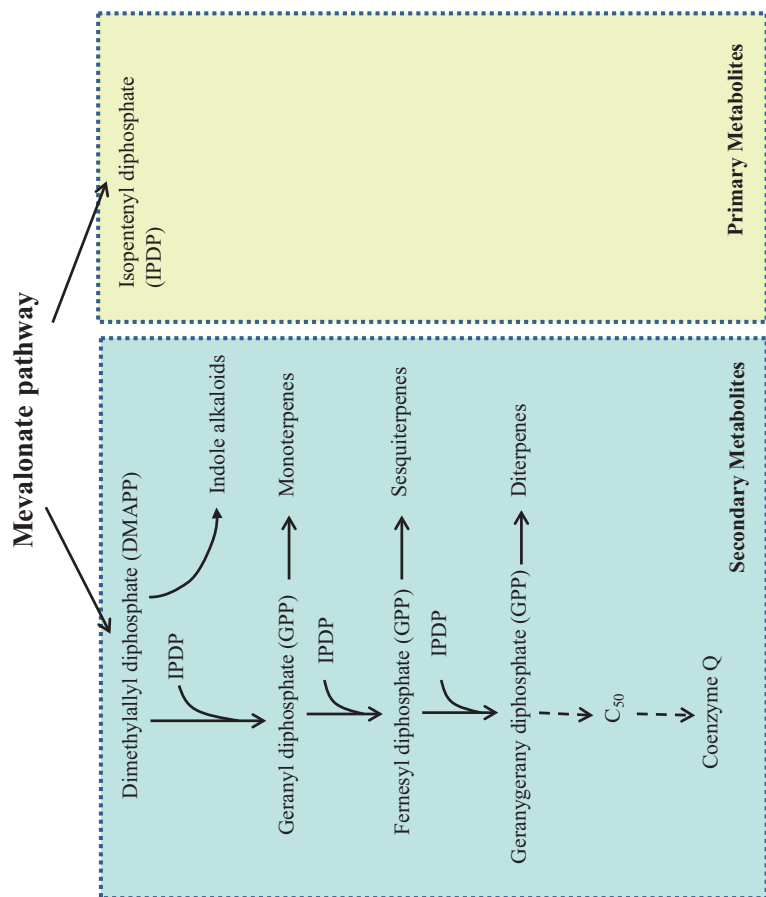


Fig. 2.3 Terpene biosynthetic pathway. Isopentenyl diphosphate and dimethylallyl diphosphate, the products of the mevalonate pathway, are the precursors of steroids, carotenoids, and coenzyme Q in many species

Terpenes are classified into three groups: monoterpenes (derived from geranyl pyrophosphate, also known as geranyl diphosphate), sesquiterpenes (derived from farnesyl pyrophosphate, also known as farnesyl diphosphate), and diterpenes/carotenoids (derived from geranyl-geranyl pyrophosphate, also known as geranyl-geranyl diphosphate) (Keller et al. 2005). Most common fungal terpenoids are carotenoids, gibberellins, and trichothecenes which act as a free-radical scavenger, plant hormone, and mycotoxin, respectively (Echavarri-Erasun and Johnson 2002; Alexander et al. 2009; Bömke and Tudzynski 2009).

2.2.4 Alkaloids

Alkaloids set up a diverse group of natural products; around 12,000 alkaloids have been identified that are most commonly produced in plants, animals, bacteria, and fungi. In the case of fungi, *Ascomycota* and *Basidiomycota* have been extensively producing the bioactive alkaloids (Guirimand et al. 2010; Xu et al. 2014). Alkaloids share the basic organic substances that contain at least one nitrogen atom in the heterocyclic ring (Mahmood et al. 2010). Alkaloids are bitter in taste and used in medical science against a diverse range of diseases and toxicants. The first microbial-derived alkaloid is ergot synthesized in *Claviceps purpurea* which causes an ergot of rye disease upon infection to the plant ovaries (Esser and Tudzynski 1978). Alkaloids accurately cannot be classified into families due to their diverse structure and precursor molecules, but based on structural similarities, the precursor molecule, and their source (Waller and Burström 1969; Anaya et al. 2006; Evans 2009; Roberts 2013; Wansi et al. 2013; Aniszewski 2015), alkaloid are separated in different types as shown in Table 2.2.

Indole alkaloids are most commonly produced in fungi which are derived from tryptophan and an isoprenoid precursor (dimethylallyl pyrophosphate). However, intermediate compounds of the shikimic acid pathway such as anthranilate, indole-3-glycerol-phosphate, 4-dimethylallyl tryptophan (4-DMAT), and tryptamine act as a precursor (Fig. 2.4) (Byrne et al. 2002; Xu et al. 2014). The synthesis of indole alkaloids is achieved by NRPS enzymes; adenylation (A) domain of NRPS activates

Table 2.2 Alkaloids classes and associated examples

S. no.	Types of alkaloids	Examples
1	Phenylalkylamine alkaloids	Ephedrine, pseudoephedrine, taxine, and hordenine
2	Pyrrolidine alkaloids	Nicotine and hygrine
3	Piperidine and pyridine alkaloids	Lobeline, coniine, and piperine
4	Tropane alkaloids	Hyoscyamine, atropine, and hyoscyne
5	Quinoline alkaloids	Quinine and quinidine
6	Indole alkaloids	Apocynaceae, Rubiaceae, and Loganiaceae
7	Purine alkaloids	Caffeine, theobromine, and methylated xanthine
8	Tropolone alkaloids	Colchicines
9	Diterpenoid alkaloids	Delcosine, lycocotonine type, and ajaconine

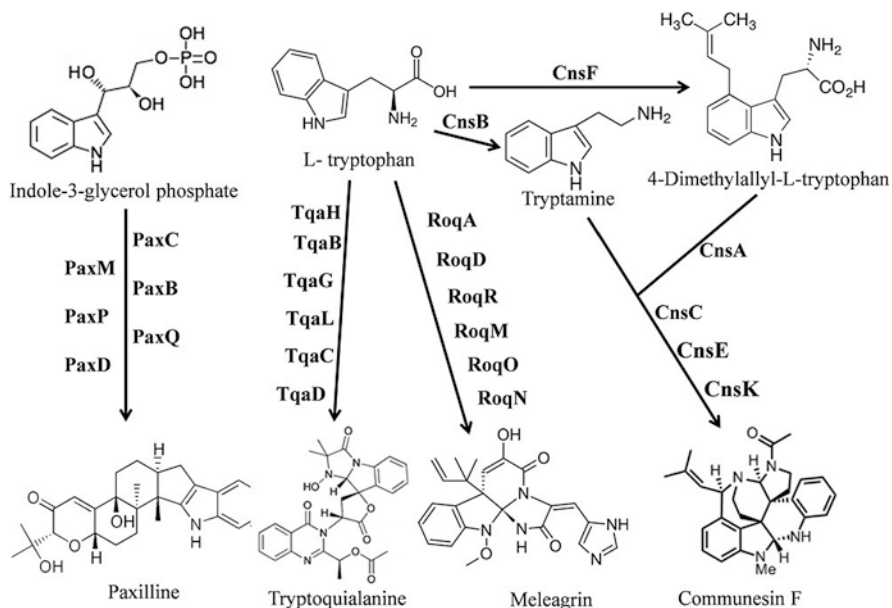


Fig. 2.4 Fungal indole alkaloid biosynthetic pathway. Paxilline biosynthesis from indole-3-glycerol phosphate in *P. paxilli* with the action of paxilline biosynthetic genes (Scott et al. 2013). Tryptoquialanine and meleagrins biosynthesis from L-tryptophan in *P. aethiopicum* and *P. chrysogenum* by respective biosynthetic genes (Gao et al. 2011; García-Estrada et al. 2011). Communesin F biosynthesis from L-tryptophan, tryptamine, and 4-dimethylallyl-L-tryptophan in *P. expansum* with respective biosynthetic genes (Lin et al. 2015)

the individual amino acids to aminoacyl-ADP, transfers to thiolation (T) domain, and forms aminoacyl thioesters. A variety of other enzymes, i.e., geranyl-geranyl pyrophosphate synthases, monooxygenases, methyltransferases, tryptophan decarboxylase, anthranilate synthase, chorismate mutase, strictosidine synthase, and tyrosine decarboxylase, are also involved in indole synthesis (Ziegler and Facchini 2008; Dubouzet et al. 2013; Xu et al. 2014). There are larger NRPS products that contain tryptophan, such as the tetrapeptide apicidin isolated from *Fusarium semitectum* that displays histone deacetylase inhibition activity (Darkin-Rattray et al. 1996; Jin et al. 2010; Xu et al. 2014).

2.3 Genetics of Secondary Metabolite Synthesis

The fungal genome sequencing projects gave insight to the SM research, thus achieving the milestone for biosynthetic gene exploration and regulation (Galagan et al. 2005). Fungal SMs are synthesized by the action of a set of enzymes which are encoded by different genes. It has been reported that the SM gene clusters are randomly distributed in the genome but most often located at the sub-telomeric regions (Fig 2.5a) (Palmer et al. 2010; Cairns and Meyer 2017). The SM biosynthetic genes

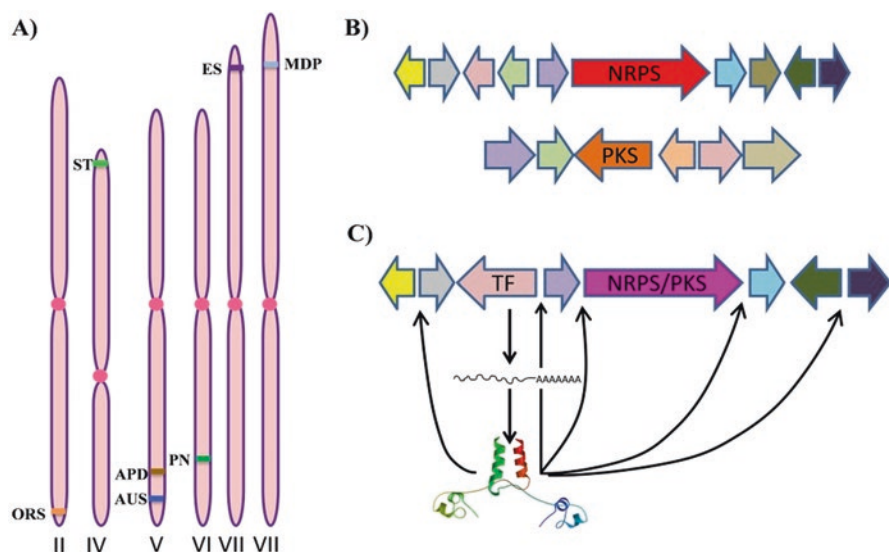


Fig. 2.5 Location, arrangement, and pathway-specific regulation of fungal SM biosynthetic genes. (a) Sub-telomeric arrangement of *A. nidulans* SM biosynthetic genes in the respective chromosomes. *ORS* orsellinic acid, *ST* sterigmatocystin, *APD* aspyridone, *AUS* austinol, *PN* penicillin, *ES* emericellamide, *MDP* monodictyphenone. (b) Physical arrangement of SM biosynthetic genes in the cluster along with NRPS/PKS. (c) Pathway-specific regulation of SM biosynthetic genes by the activation of in-cluster transcription factor

in fungi have special fetchers which are typically clustered in the genome and pooled to a single genetic locus (Fig 2.5b). The gene cluster encoded for SM is comprised of various functional genes. For example, in *A. nidulans*, each cluster contains 6–22 genes with a size ranging from 20 to 60 kb (William et al. 2005). It is expected that genes located in cluster could simplify the horizontal gene transfer and encourage the SM traits in nature (Khaldi and Wolfe 2011). It is also postulated that gene clustering could facilitate co-regulation of cluster's genes, specifically those located in the sub-telomeric regions (Palmer and Keller 2010). The cluster genes encode for NRPS/PKS, regulatory transcription factors, MFS/ABC transporters for translocation, and hydrolases and oxidases in particular for maturation of SM. Two complex proteins either non-ribosomal peptides (NRPS) or polyketides (PKS) are involved in building the general scaffolds of most SMs as described elsewhere (Palmer and Keller 2010; Brakhage 2013).

The architecture and arrangement of SM biosynthetic genes raise a question on their clustered form and sub-telomeric location. For example, there are 24 SM biosynthetic gene clusters present in the *A. fumigatus* genome. Out of 24 gene clusters, 8 were located in the sub-telomeric regions (William et al. 2005). Similarly, *Zyoseptoria tritici* contains 34 gene clusters; among them 12 are located at sub-telomeric regions (Cairns and Meyer 2017). In contrast, the sub-telomeric arrangement of the gene cluster may also influence another level of regulation incurred by

telomeric positioning effect (TPE). TPE is a phenomenon in which telomeric or sub-telomeric region genes are transcriptionally repressed due to heterochromatin formation (Gottschling et al. 1990; Castaño et al. 2005; Rosas-Hernández et al. 2008; Smith et al. 2008). TPE covers up to 30-kb region of the telomere in *A. nidulans*. The expression for aflatoxin biosynthetic gene cluster is affected by TPE (Palmer and Keller 2010). The sub-telomeric occurrence of the gene cluster is also a signal for multi-step controlled regulation and production of SMs after stopping the primary metabolism or growth. Heterochromatin starts to turn into euchromatin at stationary phase, and SM gene clusters are more amenable to transcribe and turn on the SM biosynthesis.

2.4 Regulation of Fungal Secondary Metabolites (SMs)

The SM biosynthetic genes are often silent in the initial growth phase; SM genes are transcribed continuously when cells enter in the late exponential stage to the initial decline phase. The fungal SM biosynthetic genes are arranged in a cluster form with a size up to several hundred kilo base pairs (kb) long. The regulation of SM synthesis is either governed by a single or multiple transcriptional factors (TFs). Regulation of SM synthesis in fungi occurs either at pathway-specific level or global regulation level. The in-clustered TF gene located in a pathway-specific gene cluster plays a pivotal role in the SM regulation. AfoAAflR, SirZ, and GliZ are the examples of pathway-specific regulators (Chang et al. 1995; Bok et al. 2006a; Fox et al. 2008). On the other hand, the regulatory factors localized in the genome also respond to different environmental cues for SM synthesis. VeA, PacC, LaeA, and MeaB are the known fungal global regulators that monitor the expression of SM synthesis (Yin and Keller 2011; Brakhage 2013; Knox and Keller 2015).

2.4.1 Pathway-Specific Regulatory Proteins

Pathway-specific TFs are mostly present within the SM biosynthetic gene clusters, and they tend to regulate the expression of their own biosynthetic genes (Fig. 2.5c). Pathway-specific TFs are mostly Zn2Cys6 type which are abundantly present in fungal SM biosynthetic gene clusters (MacPherson et al. 2006; Chung et al. 2013). Nonetheless, TFs belonging to Cys2His2, bZIP, and winged helix are less common (Trapp et al. 1998; Kihara et al. 2008; Chang and Ehrlich 2013; Schmitt and Kück 2000; Hong et al. 2013). *AflR*, belonging to Zn2Cys6 family TF, specifically interacts with palindromic sequence (TCGN5CGR) of the promoters and activates aflatoxin and sterigmatocystin production in *Aspergillus* spp. (Ehrlich et al. 1999). Similarly, other pathway-specific regulators such as *GliZSirZ*, *ApdR*, *CtnA*, *LovE*, and *AfoA* also interact with the promoter sequence for their activation. Gliotoxin produced from *Aspergillus*, *Trichoderma*, and *Penicillium* spp. was shown to be regulated by an in-clustered TF *GliZ* in *Aspergillus fumigatus* (Bok et al. 2006a; Fox and Howlett 2008). Deletion of *GliZ* resulted in the loss of gliotoxin biosynthesis;

however, its induced expression accelerates the gliotoxin level (Bok et al. 2006a). The SM sirodesmin PL produced from *Leptosphaeria maculans* is regulated by in-clustered TF *SirZ* (Fox et al. 2008). Moreover, *ApdR*, *CtnA*, and *LovE* regulate the biosynthesis of cytotoxic metabolite aspyridone in *A. nidulans* (Bergmann et al. 2007), citrinin in *Monascus purpureus* (Shimizu et al. 2007), and lovastatin in *A. terreus*, respectively (Van Den Berg and Hans 2011). An anti-cancerous drug asperuranone produced from *A. nidulans* was regulated by *AfoA* (Chiang et al. 2009).

Tri6 belonging to Cys2His2 zinc finger TF has a role in the regulation of trichothecene biosynthesis in *Fusarium* spp. (Proctor et al. 1995). The TFs *Bmr1*, *Cmr1*, *Cmr1p*, and *Pig1p*, present in *Bipolaris oryzae*, *Cochliobolus heterostrophus*, *Colletotrichum lagenarium*, and *Magnaporthe grisea*, respectively, belong to the Cys2His2 class that regulates the melanin biosynthesis (Tsuji et al. 2000; Kihara et al. 2008). Despite this, bZIP TFs were also reported in pathway-specific regulation in fungi. These TFs are characterized by the presence of basic and leucine zipper regions. The basic region comprises of basic amino acids (arginine and lysine) or hydrophobic residues involved in the sequence-specific DNA binding; this region is unstructured in the absence of DNA but forms an α -helical structure upon binding the DNA target site and is also considered as an essential domain. The leucine zipper (heptad repeats of leucine residues) region mediates dimerization of the protein (Amoutzias et al. 2006). TOXE, a unique TF present in the HC toxin gene cluster, regulates the HC toxin biosynthesis by interacting with TOX2 gene promoters in *Cochliobolus carbonum* (Pedley and Walton 2001).

2.4.2 Global Regulatory Proteins

It was well documented that different regulatory proteins, other than in-clustered TFs, are also involved in the regulation of fungal SM biosynthesis in response to environmental cues. These cues include carbon and nitrogen source, light, pH, and temperature (Fig. 2.6). These factors are triggered in response to SM production (Brakhage 2013; Macheleidt et al. 2016). For example, *CreA*, *AreA*, *PacC*, *LaeA*, and *HapX* are triggered in response to environmental carbon, nitrogen, pH, light, and iron starvation condition, respectively (Dowzer and Kelly 1991; Eisendle et al. 2004; Bayram et al. 2008; Schönig et al. 2008; Schrettl et al. 2010; Brakhage 2013).

2.4.2.1 Regulation by Nitrogen Source

Nitrogen is a crucial nutrient, required for growth and development. Microbes have the ability to metabolize a diverse range of nitrogen sources to colonize at diverse environmental niches and survive under nutrient limitations. Fungi most preferably utilize glutamine and ammonium, as favored nitrogen sources. In absence of these sources, non-preferred or complex nitrogen sources such as nitrate, uric acid, urea, amines, purines, pyrimidines, and amides may also be consumed (Wong et al. 2007). The selective utilization of complex nitrogenous sources is a state called nitrogen metabolite repression which activates the global regulatory circuit leading to transcriptional

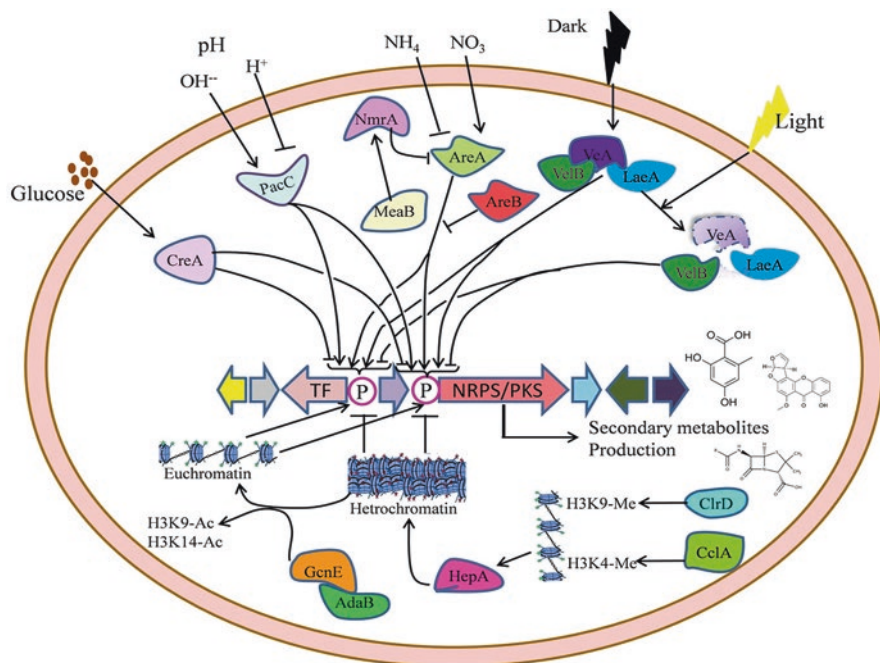


Fig. 2.6 Global regulatory proteins involved in the regulation of secondary metabolism. The NRPS- or PKS-mediated SMs are regulated in the different environmental cues such as light, pH carbohydrates, and carbon and nitrogenous source. The stimulus-responsive regulatory factors (global regulators) may also able to activate or repress the biosynthetic gene cluster of the SMs

activation of genes involved in the uptake and metabolism and ensures degradation (Marzluf 1997; Fraser et al. 2001; Magasanik and Kaiser 2002). The nitrogen metabolite repression is mediated by the GATA family TFs. Both the key regulatory genes named as *AreA* and *nit-2* in *A. nidulans* and *N. crassa*, respectively, are similar in sequence and closely related to each other. They contain Cys₂/Cys₂-zinc finger motifs, which preferentially bind to 5'HGATAR3' DNA sequence (Kudla et al. 1990; Ravagnani et al. 1997). The activity of *AreA* is regulated by both extracellular and intracellular nitrogen levels (Caddick et al. 2006). At nitrogen-limiting conditions, the level of *AreA* gets up-regulated, while it was down-regulated at nitrogen-sufficient conditions (Tudzynski 2014). In another level of regulation, *AreA* was down-regulated when it interacts with co-repressor *NmrA/Nmr1*, whereas *NmrA/Nmr1* is regulated by a bZIP TF *MeaB* in *A. nidulans*, *N. crassa*, and *F. fujikuroi* (Fig. 2.6) (Wong et al. 2007; Tudzynski 2014). *AreB* and *NreB* are also nitrogen-responsive regulators present in *A. nidulans* and *P. chrysogenum*, respectively. *AreA/NIT2* is responsible for activation of non-preferred nitrogen-metabolizing genes, whereas *AreB/NreB* acts antagonistically and represses the same set of genes, most probably via DNA-binding competition (Haas et al. 1997; Wong et al. 2009; Tudzynski 2014).

In *F. fujikuroi*, the production of growth hormone gibberellic acid and red pigment bikaverin induced under nitrogen-limiting conditions is positively regulated by AreA (Bu'Lock et al. 1974; Mihlan et al. 2003). In *F. verticillioides*, fumonisins are regulated at nitrogen-repressing condition mediated by the AreA (Kim and Woloshuk 2008; Picot et al. 2010). In *F. graminearum*, mycotoxins deoxynivalenol (DON), zearalenone, and fusarielin H are produced at nitrogen-repressing condition under the control of AreA, whereas they are slightly negatively regulated by NmrA (Giese et al. 2013). Production of aflatoxin is mediated by nitrate repression in *A. parasiticus*, whereas in *A. flavus* it is mediated by ammonium induction with response to AreA (Feng and Leonard 1998; Ehrlich and Cotty 2002).

2.4.2.2 Regulation by Carbon Source

Carbon is an essential nutrient which drastically affects the growth and development of microorganisms. Fungi preferentially utilize simple sugars such as D-glucose, sucrose, D-xylose, and acetate. Moreover, other complex carbon sources such as fructose, cellulose, xylan, pectins, alcohols, amino acids, and glycerol were also utilized under non-availability of the simple sugars (Ruijter and Visser 1997). Cells grown with the simple sugars create a carbon catabolic repression which tends to repress the transcription of a gene, involved in the complex carbon catabolism to ensure preferential utilization of glucose.

CreA/Cre1 is a C₂H₂ type of key regulatory TF protein present in *A. nidulans* and *A. niger* (Tudzynski et al. 2000) which specifically binds to the 5'-SYGGRG-3' DNA sequence in the promoters of glucose-repressible genes or complex carbon-catabolizing genes, leading to switching off of the expression in the presence of glucose (Dowzer and Kelly 1989; Flipphi et al. 2003). In *A. nidulans*, expression of xylanase (*xlnR*), cellulase, and arabinose encoding genes is also controlled by CreA (Ruijter and Visser 1997; Tamayo et al. 2008). Moreover, CreA also has a repressive role in SM biosynthesis in fungi (Fig. 2.6). The synthesis of penicillin was found to be repressed under sufficient glucose in the media, and also in response to CreA, it negatively regulates the transcription of *pcbAB*, *pcbC*, and *penDE* in *Penicillium chrysogenum* (Gutiérrez et al. 1999), not in *A. nidulans* (Espeso et al. 1993). The antibiotic pleuromutilin production was repressed in the presence of glucose, whereas it was enhanced by soybean oil in *Pleurotus mutilus* (Hu et al. 2009). Negative regulation of glucose on other SMs such as cephalosporin and lovastatin was also reported in *A. chrysogenum* and *A. terreus*, respectively (Jekosch and Kück 2000; Lai et al. 2007). However, unlike other SMs, AF production was induced by glucose (Wiseman and Buchanan 1987).

2.4.2.3 pH-Mediated Regulation

Extracellular pH is an important factor which influences the cell growth by alteration of the expression level of a variety of genes in fungi. The modulation of gene expression over a wide range of pH is controlled by the genetic regulatory system which is mediated by a global regulatory protein PacC (Caddick et al. 1986). The zinc finger PacC is constitutently expressing protein, but full-length protein is inactive and unable to activate the responsive genes. The activation of PacC is mediated by the six pal gene products at alkaline ambient pH (Fig. 2.6). In acidic conditions, the full-length PacC (72-kD) protein predominantly is in a closed conformation in *A. nidulans*, but at alkaline to neutral pH, it goes through two rounds of proteolytic cleavage and yields a

functional PacC 27 (27-kDa) TF. This PacC 27 activates the alkaline-expressed genes and represses the acid-expressed genes (Díez et al. 2002). A variety of SM biosynthetic genes are controlled by pH-mediated regulation (Fig. 2.6). In *Trichoderma virens*, 5% of the transcriptomes are pH-dependent; among these around 25% are PacC dependent, whereas secondary metabolism-related genes are predominantly regulated by PacC (Trushina et al. 2013).

SMs are mostly produced under acidic pH conditions, which are negatively regulated by PacC (Fig. 2.6). For example, a mycotoxin, fumonisin, is negatively regulated by Pac1, and its production was repressed under alkaline conditions in *F. verticillioides* (Flaherty et al. 2003). The mycotoxin, ochratoxin A, synthesized by *A. ochraceus* is another example which is synthesized under acidic pH conditions (O’Callaghan et al. 2006). On that note, the mycotoxins, aflatoxin and sterigmatocystin, induced their production by fivefold at pH 4–5 as compared to pH 8 in *Aspergillus* spp. (Keller et al. 1997). The mycotoxin deoxynivalenol (DON) regulates the production at low pH in *F. graminearum* (Gardiner et al. 2009). In contrast, PacC also positively regulates the SM production in few cases, such as penicillin biosynthesis at alkaline pH (Espeso et al. 1993).

2.4.2.4 Light-Mediated Regulation

Light is an information carrier of nature; the cellular/molecular machinery converts its electromagnetic energy (photons) into the chemical language and transmits their dynamic signals to cellular functions. The plants used it as an energy source, whereas other organisms used it as a source of information to execute biological signal for the growth and development. In fungi, light is the essential signal for asexual sporulation and induces the conidiation at red light, whereas it is suppressed at far-red light (Mooney and Yager 1990; Corrochano 2007). The reception and transmission of the light signal are modulated by the complex proteins, consisting of LOV (light-oxygen-voltage) sensing domain (that senses blue light) and PAS (Per-Arnt-Sim) domain (involved in the dimerization of proteins), which form a complex regulatory network (Ballario et al. 1998). In *N. crassa*, two major light response regulatory proteins are White Collar-1 (WC-1) and White Collar-2 (WC-2) that contain photoreceptors which dimerize by the exposure of blue light and act together as a TF complex (white collar complex, WCC). The homolog of WC-1 and WC-2 in *A. nidulans* is named as LreA (light response) and LreB (Ballario et al. 1998; Purschwitz et al. 2008). These photoreceptors activate the clock-controlled genes and regulate the circadian clock (Ballario et al. 1996; Crosthwaite et al. 1997). The LOV domain of WC-1 acts as a photoreceptor, responds to blue light, and binds to the flavin molecule (FAD or FMN). The blue light-induced conformational change eventually transduced the light signal to downstream biochemical signals (Crosson and Moffat 2002; Crosson et al. 2003). The downstream signals eventually activate the targeted proteins such as histone acetyltransferase NGF-1/Gcn5p which acetylates the H3K14 residues of light-inducible gene promoter wrap under histone proteins at heterochromatic mark (Grimaldi et al. 2006; Brenna et al. 2012). In dark conditions, WCC forms “dark” WCC which consists of WC-1/WC-2 heterodimer and binds to light-responsive elements (LREs) in the promoters of light-responsive genes, leading to the transcriptional repression (Talora et al. 1999; Froehlich et al. 2002). After elongated exposure to blue light, another photoreceptor PAS protein VIVID (VVD) accumulates to the threshold level

and disrupts the WCC complex proteins, leading to switching off of the light-dependent transcription (Schwerdtfeger and Linden 2001, 2003). VVD is a white collar-dependent blue light photoreceptor which represses the light responses and also deals with the changes in light intensities (Schwerdtfeger and Linden 2001, 2003). The disruption of WCC complex is mediated by the conformational change of WC-1 protein by phosphorylation reaction leading to switching off of the gene (Schwerdtfeger and Linden 2000; He and Liu 2005). Sterigmatocystin (ST) biosynthesis in *A. nidulans* was suppressed on exposure of blue or white light and induced on red light exposure greater than the dark condition (Purschwitz et al. 2008).

Velvet (VeA) Family of Light-Responsive Global Regulators

Proteins under velvet (VeA) family are abundantly distributed in ascomycetes and basidiomycetes and play a vital role in the secondary metabolism (Ni and Yu 2007). VeA is a light-dependent regulatory protein; exposure of light governs its localization within the cell. During dark conditions, VeA accumulates in the nucleus and upon exposure of light mobilizes it to the cytoplasm (Stinnett et al. 2007; Bayram et al. 2008). The truncated VeA mutant protein does not respond to the light signals and is effectively unable to synthesize mycotoxin ST and antibiotic penicillin (Kato et al. 2003). VeA also has a crucial role in conidia formation, which is induced by red light (Mooney and Yager 1990). The heterotrimeric velvet complex VelB/VeA/LaeA is also involved in the light-dependent regulation of cellular developmental and secondary metabolism in *A. nidulans*. Upon light exposure, the cellular VeA level and its translocation to the nucleus both get reduced (Fig. 2.6).

The VelB (another developmental regulator like VeA) forms the dimer with VeA (VelB-VeA) in the cytoplasm and transports to the nucleus. VeA acts as a bridge between VelB and a secondary metabolism nuclear master regulator, LaeA. The deletion of either VeA or VelB results in impaired sexual fruiting body formation and SM production (Fig. 2.6) (Bayram et al. 2008; Purschwitz et al. 2008). The similar trimeric complexes have also been reported in other fungal species such as *F. oxysporum* and *P. chrysogenum* (Kopke et al. 2013; López-Berges et al. 2013). Other than these, VosA protein also mediates asexual differentiation via heterodimer formation with VeA or VelB. The heterodimer (VosA-VelB) formation results in inhibition of asexual differentiation in the dark and induces trehalose biosynthesis in spores to protect from stress (Ni and Yu 2007). In *A. nidulans* a sexual development positive regulator, VelC, has also been recently characterized (Park et al. 2014). FphA is another light-responsive transcription regulatory protein which senses red light. It interacts with the VeA, induces the asexual spore formation, and represses the fruiting body formation (Blumenstein et al. 2005; Purschwitz et al. 2009). LreA and LreB act as activators of the sexual cycle and are negatively regulated by light with the action of FphA (Purschwitz et al. 2008).

2.4.2.5 Chromatin-Mediated Regulation

The eukaryotic gene expression and SM biosynthesis are also controlled by another hierarchical level of regulation known as chromatin-mediated regulation. This primarily depends on the degree of chromatin compaction or chromosomal organization of biosynthetic genes. The fungal SM biosynthetic gene clusters are most often present

in the telomeric region and usually not expressed in the normal conditions (Reyes-Dominguez et al. 2010; Strauss and Reyes-Dominguez 2011). Telomeres of the chromosomes are normally heterochromatic in nature, which get relaxed into the euchromatic form by the chemical modifications of nucleosomal histone amino acids. These modifications at the epigenetic level dramatically monitor the expression of SM biosynthetic genes (Bok et al. 2009). The majority of chemical modifications are achieved at the basic amino acids of histone proteins located in the nucleosome.

Histone Modifications

The large eukaryotic genome (10–100,000 Mb) is systematically compacted to several thousand folds in the nucleus (Hodgkin 2001). This compaction is mediated by histone proteins which bind to the DNA molecule in a systematic manner and are organized in the chromatin structure (Kornberg 1974). Chromatin consists of a repetitive unit of nucleosome which comprises of 147 bp of DNA molecule, which is wrapped around an octameric histone protein (H3/H4 and H2A/H2B) and thereby forms an 11-nm fiber structure (Thomas and Kornberg 1975; Luger 2003). The thread of nucleosome is further condensed into a 30-nm fiber by linker histone H1 (Brosch et al. 2008). The various associated proteins further condense to higher hierarchical level and form compact chromatin structure. Certain segments of the entire chromosomes become more condensed than the rest of the sections; the condensed region is termed as heterochromatin and the relaxed region as euchromatin. The heterochromatic state is mostly present at the telomeric and centromeric regions of the chromosome, whereas the euchromatic state is present in the rest of the region. The heterochromatic state prevents the transcription of genes, whereas the euchromatic state facilitates to interact with TFs (Sterner and Berger 2000). These core histones (H2A, H2B, H3, and H4) are conserved throughout the eukaryotes; these consist of a globular domain and versatile N-terminal tail (Luger 2003). N-terminal tail can be easily modified by the action of various post-translation modification enzymes as acetylation, phosphorylation, methylation, ubiquitination, ADP-ribosylation, and SUMOylation (Verdone et al. 2006; Brosch et al. 2008; Martinez-Zamudio and Ha 2012; Rossetto et al. 2012; Wilson and Hochstrasser 2016). However, methylation and acetylation have been widely involved in the chromatin remodeling and secondary metabolite biosynthetic gene regulation (Gacek and Strauss 2012).

Histone Methylation

Histone methylation inferred the transfer of a methyl group from S-adenosyl-L-methionine (SAM) to substrate proteins or specific amino acid residue. Methylation of histone proteins is specifically restricted to lysine and arginine at different positions and represents a much more complex language as compared to acetylation. The methylation of some residues such as H3K9, H3K27, and H3K4 is best studied in SM production (Fig. 2.6). Acetylation and methylation events on lysine do not occur simultaneously on the same residue (Gacek and Strauss 2012). The H3K9 in *A. nidulans* is strongly methylated by methyltransferase ClrD; di- and trimethylation of H3K9 subsequently recruits HepA and forms the condensed heterochromatin (Bannister et al. 2001; Reyes-Dominguez et al. 2010). In contrast, deletion of

ClrD in *A. nidulans* decreased the trimethylation state of H3K9 and subsequently resulted to loss of HepA recruitment in the ST cluster. Furthermore, deletion of HepA decreased the heterochromatin formation and increased the AflR expression (Reyes-Dominguez et al. 2010). In addition, H3K4 methylation is also associated with SM production; it occurred by CclA gene in *A. nidulans*. The deletion of CclA resulted in the reduction of H3K4 methylation along with H3K9 trimethylation in some SM gene promoters which increases the production of SMs F9775B and F9775A and expression of their associated genes (Fig. 2.6) (Bok et al. 2009). However, methyltransferase LaeA directly/indirectly induces the secondary metabolism and virulence in the *A. nidulans* and neutralizes the heterochromatic mark establishment. Though LaeA contains the methyltransferase domain, substrate or methyl-accepting residue has not been identified up to date, whereas it self-methylates methionine 207 in *A. nidulans*. Furthermore, self-methylation of methionine 207 did not show any role in its biological function (Patananan et al. 2013). The LaeA knockout strain of *A. nidulans* down-regulates the expression of ST, penicillin, and some indole alkaloid biosynthesis gene clusters in microarray experiments (Bok et al. 2006b; Yin and Keller 2011). Furthermore, LaeA knockout strain of *A. fumigatus* up-regulates the 22 secondary metabolism gene clusters and down-regulates the 13 SM gene clusters (Perrin et al. 2007).

Histone Acetylation

Acetylation of histone proteins under distinct physiological conditions also plays an important role in the regulation of secondary metabolism gene clusters. Histone acetylation marks are subjected to active transcription of genes at several positions; it occurs by histone acetyltransferase (HAT) enzymes, and these marks were removed by histone deacetylases (HDAC). The hyperacetylation of histone commonly responds to euchromatin formation, while hypoacetylation responds to heterochromatin formation and silences the gene transcription. Few histones involved in acetylation such as H3K9, H3K14, and H4K12 greatly influence the SM production and are acquired by multi-subunit Saga/Ada complexes or other associated histone acetyltransferases (Lan et al. 2016).

The GcnE and AdaB histone acetyltransferases are a part of SAGA-ADA complex and acetylate the H3K9 and H3K14 residue at the promoter region of orsellinic acid biosynthesis genes in fungal and bacterial interaction in *A. nidulans*. However, this acetylation mark is also detected in genes other than secondary metabolism gene clusters (Nützmann et al. 2011). In addition, the regulatory subunits of Saga/Ada, GcnE, and AdaB, respectively, are required for induction of conidiation in *A. nidulans* (Cánovas et al. 2014). Furthermore, the acetylation of H3K9 which enhances the orsellinic acid and sterigmatocystin biosynthesis gene clusters is triggered by *S. rapamycinicus* (Reyes-Dominguez et al. 2008). Targeted mutation of the potentially acetylated lysine residues 9, 14, 18, and 23 of histone H3 led to major changes in the transcriptional and metabolite levels of sterigmatocystin, orsellinic acid, and penicillin in *A. nidulans* (Nützmann et al. 2013). EsaA acetylates the H4K12 and enhances the sterigmatocystin, penicillin, terrequinone, and orsellinic acid production (Soukup et al. 2012). Furthermore the SM production is also

enhanced by histone deacetylase (HDAC) inhibitor such as suberoylanilide hydroxamic acid (SAHA), trichostatin A, and 5-azacytidine (Fisch et al. 2009; Lim et al. 2012; Zutz et al. 2013; Albright et al. 2015), whereas SM production is blocked by histone acetyltransferase HAT inhibitor anacardic acid and curcumin (Netzker et al. 2015; Wee 2015).

For the activation of cryptic SM gene clusters, HDAC inhibitors (HDACi) have been widely used in fungi. These HDACi are valproic acid and trichostatin A (TSA), and suberoylanilide hydroxamic acid (SAHA) treatment effectively increases the SM production by chromatin modification in fungi (Henrikson et al. 2009; Zutz et al. 2013). In addition to the HDACi, histone demethylase inhibitor such as 5-azacytidine is also used in the SM modulation (Zutz et al. 2013).

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