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MINI-REVIEW

Genome editing approaches: manipulating of lovastatin and taxol synthesis of filamentous fungi by CRISPR/Cas9 system

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Abstract Filamentous fungi are prolific repertoire of structurally diverse secondary metabolites of remarkable biological activities such as lovastatin and paclitaxel that have been approved by FDA as drugs for hypercholesterolemia and cancer treatment. The clusters of genes encoding lovastatin and paclitaxel are cryptic at standard laboratory cultural conditions (Kennedy et al. Science 284:1368–1372, 1999; Bergmann et al. Nature Chem Biol 3:213–217, 2007). The expression of these genes might be triggered in response to nutritional and physical conditions; nevertheless, the overall yield of these metabolites does not match the global need. Consequently, overexpression of the downstream limiting enzymes and/or blocking the competing metabolic pathways of these metabolites could be the most successful technologies to enhance their yield. This is the first review summarizing the different strategies implemented for fungal genome editing, molecular regulatory mechanisms, and prospective of clustered regulatory interspaced short palindromic repeat/Cas9 system in metabolic engineering of fungi to improve their yield of lovastatin and taxol to industrial scale. Thus, elucidating the putative metabolic pathways in fungi for overproduction of lovastatin and taxol was the ultimate objective of this review.

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Introduction

Filamentous fungi are ubiquitous microorganisms inhabiting the entire environmental niches with positive and negative impacts on human health and life. Fungi that have an elaborated secondary metabolism ranged from mycotoxin and carcinogenic metabolites to medically relevant anticancer, cholesterol-lowering statins, and immunosuppressive metabolites (Moretti et al. 2013; Wu et al. 2014). Fungal secondary metabolites (SMs) are heterologous low molecular weight molecules giving the fungus advantages for competition over the surrounding ecosystems (Berdy 2005); these compounds are in continuous evolution over the hundreds of years with the environmental dynamic complexity (Brakhage 2013). More than 45,000 SMs with antimicrobial and therapeutic activities have been recovered and characterized from filamentous fungi (Bladt et al. 2013). Fungal SMs have been categorized into different types based on their structure and biosynthesis such as polyketides, non-ribosomal peptides, and terpenoids (Crawford and Townsend 2010; Hertweck 2009). Statins and taxol are the eminent bioactive polyketides and terpenoids from fungi (Bladt et al. 2013; Chen et al. 2014; Liao et al. 2012); thus, we have focused on metabolic engineering of these compounds from fungi.

There is a massive indication for a novel bioactive scaffold compounds from fungi; however, exploring of new active compounds mainly depends on traditional screening of fungi from unusual sources, eliciting their secondary metabolism using various nutritional, cultural conditions, and inhibitors. Majority of biosynthetic gene clusters of SMs are silent under standard

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laboratory conditions (Cueto et al. 2001). Induction of the secondary metabolites by traditional methods was frequently documented; however, the laborious work and the lower yield of target compound are the main technological challenges.

Recently, genome editing was developed to precisely manipulate the synthesis of polyketides from *Aspergillus fumigatus* (Fuller et al. 2015), *A. nidulans*, *A. aculeatus* (Nodvig et al. 2015), and *A. oryzae* (Katayama et al. 2016) as describe in details on this review. Genome engineering using site-specific nuclease (SSN) inducing a targetable DNA double-stranded breaks (DSBs) has been emerged as a reliable molecular tool to gene knockout and knock-in to enhance the production of target metabolites (Li et al. 2013). DSBs of DNA are common responses due to the spontaneous mechanical stress such as exposure to ionizing radiations, radiomimetic chemicals, transgenic DNA, or crossing over during meiosis division (Britt 1999). Homologous recombination (HR) and non-homologous end joining (NHEJ) are the two common endogenous mechanisms for repairing of DNA DSBs (Bortesi and Fischer 2015). HR is a precise, high-fidelity repairing mechanism that used homology template or exogenous DNA fragment for target gene modification or gene insertion (Bortesi and Fischer 2015); thus, it is designated as error-free DNA repair (Paques and Haber 1999). HR repair of DSBs mostly occur at S or G2 stages of cell cycle during the contiguity of sister chromatids; however, NHEJ is independent on presence of homologous chromosome function throughout the whole cell cycle. Thus, it is the major frequent pathway (Sonoda et al. 2006). NHEJ is an error-prone repair system that depends on random insertions or deletions (indels) of nucleotides, causing frame shift mutations and, thus, effectively creating gene knockout (Kim and Kim 2014; Steentoft et al. 2011) (Fig. 1). NHEJ is the most common DSB repairing mechanism in microorganisms, comparing to the lower target integration by HR (Puchta 2005). Three enzymatic activities are essential for NHEJ repairing mechanism: nuclease to exclude the damaged nucleotide, polymerase to repair, and ligase to restore the phosphodiester bond (Lieber 2008). Overall, NHEJ is the most conserved mechanism in fungi for chromosomal DSBs (Carvalho et al. 2010). HR repairs depends on Rad52 proteins that mediates targeted integration between two homologous DNA sequences, while NHEJ pathway is mediated by activities of Ku heterodimer (Ku70/Ku80-protein complex) and DNA ligase IV to ligate DSBs without any homology (Shrivastav et al. 2008). The frequency of HR repairs in filamentous fungi is extremely lower than in *Saccharomyces cerevisiae*. However, inactivation of Ku70/Ku80 proteins as a component of NHEJ mechanism in *Neurospora crassa* induces the frequency of HR mechanism up to 100% (Meyer 2008). Similarly, knockout of *akuA* gene in *A. fumigatus* encoding Ku70 protein that mediates the NHEJ indirectly mediates the HR repairs (Krappmann et al. 2006).

Acknowledging to the endogenous DNA repairing mechanisms (HR and NHEJ), various technological tools have been inspired to precisely induce DSB of a target gene by exogenous nucleases, then mediating repairing through insertion, deletion, or replacement of specific sequence. Several genome editing technologies have emerged, namely, meganucleases, zinc finger nucleases (ZFNs), and transcriptional activator-like effector nucleases (TALENs) (Miller et al. 2007; Wood et al. 2011). These technologies are based on artificial bipartite enzyme having modular DNA-binding domain and Fok1 nuclease, in which the DNA binding domain can be engineered to recognize specific DNA sequence (Boch et al. 2009; Jankele and Svoboda 2014; Zhang et al. 2011). These metabolic engineering strategies have been successfully used in various microbes and plants; however, the difficulty to engineer these nucleases is the major limitation that reduces their successes (Voytas 2013). Recently, clustered regulatory interspaced short palindromic repeat (CRISPR) system (Jinek et al. 2012) based on RNA-guided engineered nuclease (Cas9) has emerged as an extraordinary genome editing technology in microbes.

In this review, the different nuclease-based genome editing technologies implemented for metabolic engineering of filamentous fungi were discussed. Genomics of lovastatin and taxol synthesis by fungi were described, emphasizing the prospective role of CRISPR/Cas9 technology for triggering the biosynthesis of these metabolites.

Genome editing technologies

Meganucleases

Meganucleases are group of endonucleases that have been used as genome editing tool for more than 10 years ago (Pingoud and Silva 2007). Based on their conserved amino acid sequences, there are five families of meganucleases, "homing endonucleases" (Stoddard 2011; Zhao et al. 2007); LAGLIDADG endonuclease is the most common family in living organisms that nominated as selfish genetic elements (Silva et al. 2011). These endonucleases are encoded by mobile DNA elements inserted within the genomes of eukaryotes, prokaryotes, and viruses (Gimble et al. 2003) (Fig. 2). The LAGLIDADG enzymes have two functions as RNA maturase catalyzing the splicing of its introns and specialized endonuclease recognizing and catalyzing the exon-exon junction sequence wherein their intron reside, designated as homing endonuclease (Gogarten and Hilario 2006). There are two structurally different types of LAGLIDADG proteins: single-motif (I-CreI) and double-motif protein (PI-SceI) (Duan et al. 1997; Heath et al. 1997) that precisely recognized DNA sequence ranging from 14 to 40 bp. PI-SceI is a yeast LAGLIDADG endonuclease (50 kDa) that developed from

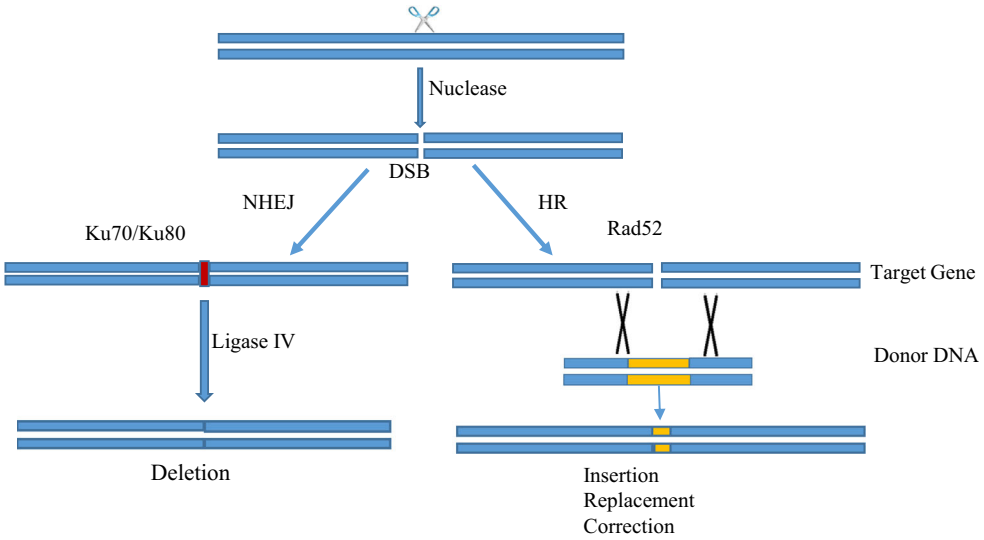

Fig. 1 Repairing mechanisms of nuclease induced double-stranded breaks (DSBs) of DNA, non-homologous end joining (NHEJ), and homology recombination (HR) pathways. NHEJ-mediated repair can insert and/or delete some few nucleotides (indel mutation). HR-mediated repair can introduce precise point mutations, by corrections or insertions or replacement, based on the donor DNA template

the intein of vacuolar H^+-ATPase catalytic subunits (Gimble and Thorner 1993). The activity of this endonuclease is dependent on Mg^{2+} with recognition sequence of 31 bp, yielding 4-bp 3′-overhang extension (Wende et al. 1996). However, there are different proteins from the LAGLIDADG family that have similar tertiary structure, encoded from unique introns, namely, I-CreI is a single-motif homodimer encoded by group I introns (Heath et al. 1997), I-*Dmo*I is a dual-motif monomeric proteins encoded by archaeal introns (Silva et al. 2011; Silva et al. 1999), and PI-*Pfu*I monomeric proteins are archaeal intein (Ichiyanagi et al. 2000). Meganucleases has been used for various genome editing applications, targeting the

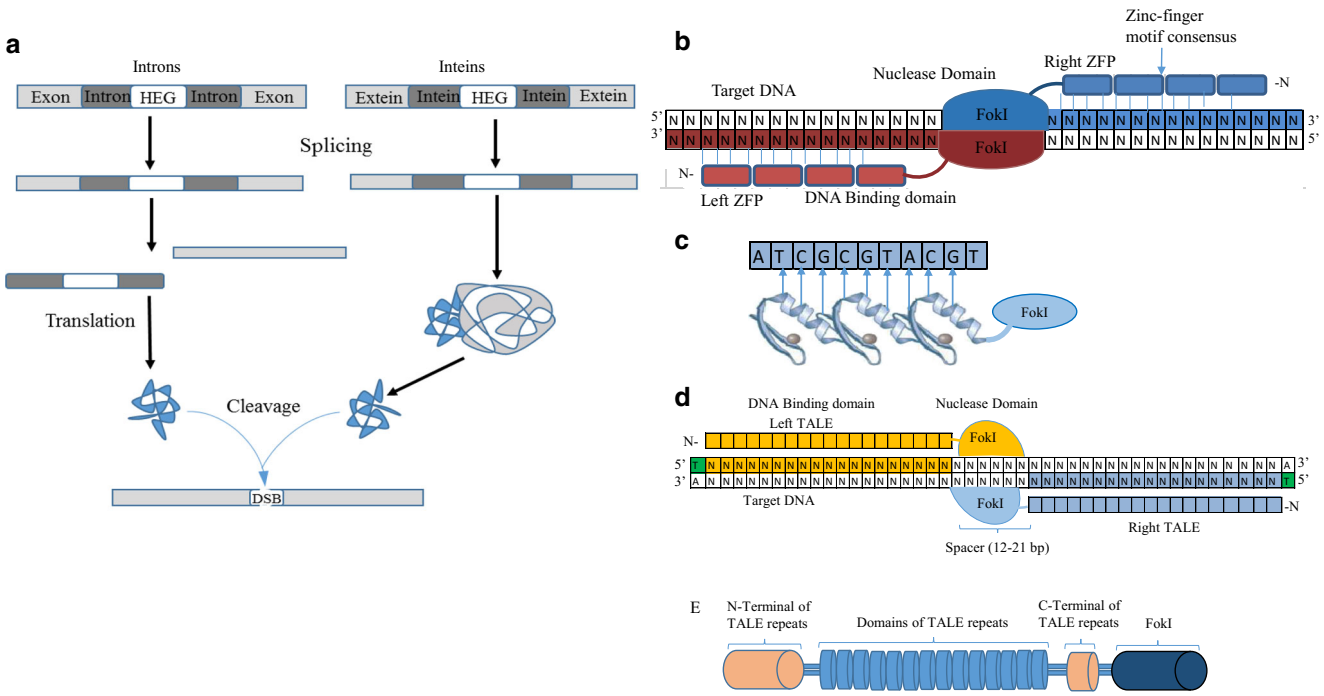

Fig. 2 Overall view of different genome editing technologies. **a** Meganuclease structure; expression of homing endonuclease gene (HEG) from the mobile group I introns and inteins, processing to endonuclease for cleavage of target DNA by DSBs. **b** Structure of ZFN. ZFN dimer bound to its target DNA, each one has zinc-finger protein (ZFP) at the N-terminus and FokI nuclease domain C-terminus. Zinc-finger motif consensus represented by CX2-4CX3FLX2HX3H, which X represents any amino acids. **c** There are three to six arrays of zinc finger proteins that is ranged to recognize the target sequence. The target sequence for ZFN pair is typically 18–36 bp in length excluding the spacers. **d** Structure of TALEN. Pair of TALENs targets the DNA; each construct has TALE protein domain at the amino acid terminus and FokI at carboxyl terminus. The left TALE and right TALE repeat domains recognized 30–40 bp of the target DNA; each repeat has 33–35 amino acids that recognize one single base via its repeated variable diresidues (RVDs). **e** Diagram of TALEN showing the TALE repeat domain with the N-terminal and C-terminal binding FokI nuclease (modified from Kim and Kim 2014)

desired genes by induction of site-specific, double-stranded breaks, then repairing via NHEJ and HR mechanisms (Fig. 2a) (Silva et al. 2011).

ZFNs

ZFN is a site-specific endonuclease that precisely bind and cleavage target DNA at specific recognition sequence. Structurally, ZFNs have two domains: DNA-binding zinc finger protein (ZFP) and *Fok*I restriction endonuclease domain (Kim et al. 1996). *Fok*I is a type II restriction endonuclease isolated from *Flavobacterium okeanokoites* having N-terminal DNA-binding domain and C-terminal DNA cleavage domain (Durai et al. 2005). Once the DNA-binding domain (ZFP) recognized the 5′-GGATG-3′ sequence, the DNA-cleavage nuclease is activated and cleaves non-specifically the sense strand after 9 nucleotides downstream and antisense strand at 13 nucleotides upstream to the recognition site (Fig. 2b). *Fok*I recognizes the non-palindromic sequences 5′-GGATG-3′ and 5′-CATCC-3′ in DNA duplex and cleavage at 9/13 nt downstream of the recognition site (Szybalski et al. 1991), with no cleavage at the site of recognition. ZFNs combine the non-specific cleavage domain of *Fok*I endonuclease based on ZFP mediation to the target sequence to make DSBs. Once the DNA-binding domain is anchored to the recognition site, the nuclease domain is substantially activated through allosteric signals to cleavage the target. Programming of ZFNs depends on the reliable designing of ZFPs that can specifically recognize the target within genome (Durai et al. 2005). The active ZFN is a monomer, but it can be dimerized based on other ZFP domain; thus, using dimer of ZFN can bind simultaneously to adjacent recognition half-sites that separated by spacers of 5–7 bp (Fig. 2b) (Bitinaite et al. 1998). This dimerization doubles the strength of recognition sites and increases the *Fok*I specificity to mediate precise DSB at the target genome (Bitinaite et al. 1998). The feasibility of engineering of chimeric *Fok*I endonuclease by fusion with other DNA-binding domains makes ZFN a versatile genome editing tool. The specificity of ZFNs is mainly determined by ZFPs that consist of tandem array of Cys_2His_2 zinc finger, which is the most common DNA-binding motif in eukaryotes. Each zinc finger motif can recognize a 3-bp DNA target sequence; practically, 3–6 zinc finger motifs are used to generate a single ZFN subunit that binds to target DNA sequence of 9–18 bp. The recognition DNA sequence 18 bp can confer the specificity of ZFN technology within 68 billion bp of human genome (Beerli et al. 2000). Each ZFP motif has about 30 amino acids and folded into $\beta\beta\alpha$ structure which is stabilized by chelation of zinc within the conserved Cys_2His_2 residues (Durai et al. 2005). The ZFP motifs bind to substrate by inserting the α-helix surface amino acids into the major groove of DNA double helix via triplet codons (Pavletich and Pabo 1991) (Fig. 2c). However, for the complex nature of interacting ZFP with the target DNA, designing the precise targeted ZFN is a challenge (Sander et al. 2011).

TALENs

TALENs are fusion of the *Fok*I cleavage domain at C-terminal and DNA binding domain from transcriptional activator-like effector (TALE) proteins (Gaj et al. 2013). TALENs is much similar to ZFN technology; however, the DNA binding domains are TALEs, naturally derived from *Xanthomonas* spp. and *Ralstonia* spp. (Doyle et al. 2012; Li et al. 2011), that composed of 33–35 amino acid repeat domains, each of which recognize a single base pair in the major groove of DNA substrate (Deng et al. 2012; Mak et al. 2012). The specificity of each repeat domain is determined by the two amino acids at positions 12 and 13 that are designated as repeated variable diresidues (RVDs) (Fig. 2d) (Mak et al. 2012). Each TALE domain has four different RVD modules, namely, Asn-Asn, Asn-Ile, His-Asp, and Asn-Gly that recognize guanine, adenine, cytosine, and thymine, respectively (Kim and Kim 2014). The single base recognition of TALE-DNA binding repeats affords great flexibility than triplet-confined zinc-finger proteins; however, cloning of TALE arrays is an elevated technical challenge due to the extensive identical repeats. TALE in *Xanthomonas* spp. is highly conserved domain that is injected to the host plant via type III secretion system, then binds to genomic DNA, altering the host gene transcription, thus facilitating the bacterial colonization (Boch and Bonas 2010). The TALE repeats (33–35 amino acid repeat) are flanked by N-terminal and C-terminal domains (Fig. 2e), with conserved thymine (T) as the first base of ZFP repeats downstream to the 5′-terminus (Joung and Sander 2013). The specificity of TALE repeats for binding with target DNA depends on identity of the hypervariable residues (RVD). However, manipulation of TALE repeats is the challenge for determination of TALEN specificity for genome editing purposes.

RNAi

RNA interference (RNAi) is the common gene silencing mechanism in eukaryotes, which 20–30 nt of small non-coding RNA (ncRNA) act as regulators for RNA stability, host defense, transcription, and translation (Carthew and Sontheimer 2009; Ding and Voinnet 2007). Active RNAi requires an Argonaute/P-element induced wimpy (Piwi) protein as the core component of RNA-induced silencing complex (RISC) (Hock and Meister 2008), guiding ncRNA to the target gene (Carmell et al. 2002; Cerutti and Casas-Mollano 2006). The family of Argonaute proteins has two subfamilies: Ago (Argonaute) that is ubiquitously expressed in eukaryotes and Piwi that is expressed in germ lines in humans (Carmell et al. 2002; Hutvagner and Simard 2008). Argonaute proteins are the key players in gene-silencing mechanisms guided by

small ncRNA. There are three categories of RNAi in eukaryotes, namely, short interfering RNAs (siRNAs), microRNAs (miRNAs), and Piwi-interacting RNA (piRNA) (Hock and Meister 2008). Biosyntheses of siRNA and miRNA are derived from double-stranded RNA (dsRNA) by action of Dicer, generating short duplex (21–25 nt). siRNAs are derived from exogenous dsRNA, endogenous transcript, from long hairpin transcripts (Ghildiyal and Zamore 2009), that require RNA-dependent RNA polymerase (RdRP) to generate dsRNA from single-stranded RNA (ssRNA) (Dang et al. 2011; Gent et al. 2010), that completely matched to mRNA target. While, miRNAs are generated from miRNA-encoding genes that generate ssRNA that form hairpin structure. RNAi has three components: Dicer, Argonaute, and RdRP proteins identified in eukaryotes, ensuring the involvement of RNAi as common regulatory mechanism in all eukaryotes (Dang et al. 2011). In RNAi biogenesis, the aberrant RNA is recognized and converted into dsRNA by RdRP, subsequently processed by Dicers into siRNA, then loaded to Argonaute proteins (Nakayashiki et al. 2005). In filamentous fungi, RNAi is the common defense mechanism against viral and transposon invasion (Dang et al. 2011) (Fig. 3). The expression of dsRNA in *N. crassa* triggers a robust dsRNA-activated transcriptional program that subsequently activates the expression of all RNAi genes including Ago proteins, Dicer as antiviral mechanisms (Dang et al. 2011). In asexual stages of *N. crassa*, RNAi silencing phenomena "quelling" occurred by introduction of repetitive DNA sequences triggering the post-transcriptional gene silencing (Fulci and Macino 2007). Quelling is the most common gene silencing mechanism in *Ascomycetes* at the post-transcriptional level, in which the generated siRNA targets the mRNA to be silenced (Fulci and Macino 2007); it is controlled by qde-1 (encodes RdRP), qde-2, qde-3, and Dicer proteins (Catalanotto et al. 2000). However, in sexual stage, there are two silencing mechanisms, namely, repeated-induced point (RIP) mutations and meiotic silencing by unpaired DNA (Selker and Stevens 1987; Shiu et al. 2001).

miRNA is a common regulator to the endogenous genes, while siRNA is the defender of genome integrity against invasive nucleic acids of virus and transposons (Carthew and Sontheimer 2009). Single strand of siRNA (siRISC) and miRNA (miRISC) are associated with the RISCs (Carthew and Sontheimer 2009; Hammond et al. 2000); for both types, the target gene to be silenced are specified by RNA Watson-Crick base pairing. The main differences between miRNA and siRNA are the following: miRNAs are endogenous from the own cell genome, processed from stem-loop precursors with incomplete dsRNA, while siRNAs mainly originated exogenously from virus, transposons, and excised from long, fully complementary dsRNA (Tomari and Zamore 2005). The biogeneses of siRNA and miRNA are illustrated in Fig. 3.

The siRNA guide strand directs the RISC for perfect pairing with target RNA that subsequently degraded. RNA degradation is induced by Piwi domain of Ago protein, "Slicer" activity cleaving the phosphodiester bond between the target nucleotide that base paired to siRNA residues 10 and 11 (counting from the 5′ end) generating product with 5′-monophosphate and 3′-hydroxyl termini (Carthew and Sontheimer 2009; Tomari and Zamore 2005). Once doing this initial cut, the cellular exonuclease cleaves the fragment to complete the degradation process (Orban and Izaurralde 2005) and the RISC cleavage product is further used for oligouridylation, triggering the exonucleolytic activity (Shen and Goodman 2004). The cleavage products were released, and the RISC becomes free for additional targets (Carthew and Sontheimer 2009).

Dicer (endoribonuclease Dicer, helicase with RNase motif) is a member of RNase III family that cleaves the dsRNA and pre-miRNA into short dsRNA fragments siRNA and miRNA, respectively (Agrawal et al. 2003). RNase III members are few nucleases that precisely cleave the dsRNA into 3′-overhang of 2–3 nt of hydroxyl termini and 5′phosphate (Agrawal et al. 2003). This enzyme was firstly identified in *Drosophila* extract that has the ability to produce fragments of about 22 nt, initiating the RNAi processing (Bernstein et al. 2001). Dice has four domains: amino terminal helicase domain, dual RNase III motif, dsRNA binding domain, and PAZ domain (Piwi, Argonaute, Zwille domain) which is shared with RDE1/QDE2/Argonaute family (Catalanotto et al. 2000; Tabara et al. 1999). Argonaute proteins are the core of RNA silencing system, Ago clade, associated with miRNA and siRNA (Meister and Tuschl 2004; Tomari and Zamore 2005).

CRISPR/Cas9 system

CRISPR-associated, RNA-guided DNA endonuclease (Cas9) is the latest genome editing technology that has been discovered in bacteria as unique defense mechanism against invading phages (Sorek et al. 2013). Approximately 50% of bacteria and 90% of archaeal genome sequences possess the CRISPR/Cas system loci to confer their resistance to foreign invading DNA elements and phages (Makarova et al. 2011). This adaptive immune system protects bacteria from invading virus and horizontal gene transfers (Horvath and Barrangou 2010). CRISPR system was firstly identified as repeated sequence downstream to the intestinal alkaline phosphatase gene (Ishino et al. 1987) that is derived from invasive DNA of bacteriophages (Mojica et al. 2005), associated with *cas* genes that are in proximity to CRISR loci (Bolotin et al. 2005). The CRISPR loci are transcribed and processed into CRISPR RNA (crRNA) that subsequently guide the endonuclease Cas9 to mediate precise sequence-specific cleavage of foreign DNA (Heintze et al. 2013). This system of adaptive immunity functions to memorize and eliminate the pathogen.

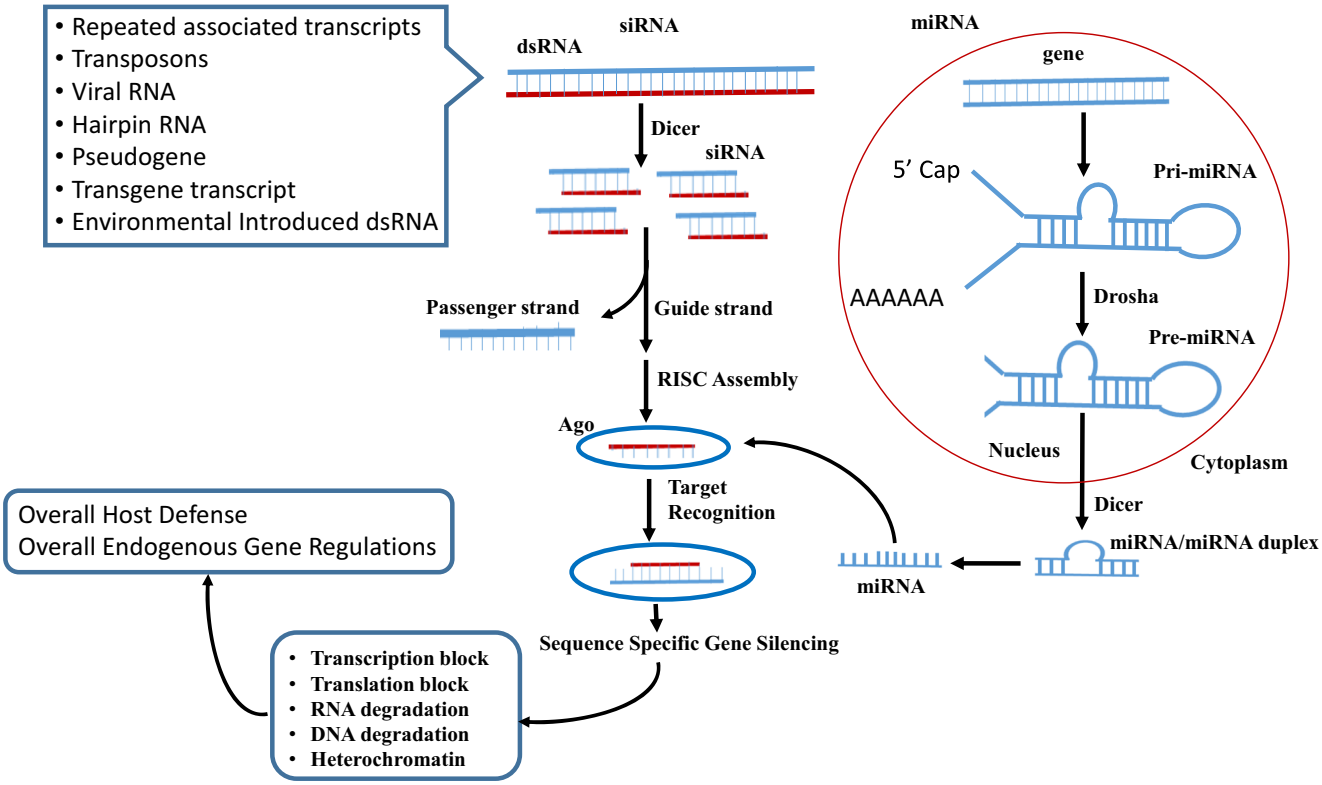

Fig. 3 Biogenesis of siRNA and miRNA and the mechanism gene silencing. Different sources of RNA can be adopted as dsRNA by RDRP. The dsRNA can be processed by Dicer to siRNA. siRNA are 20–30 nt that loaded into the Argonaute proteins; the guide strand of siRNA is further assembled into the RISC (siRISC), while the passenger strand is degraded. The guide strand on siRISC targets the RNA by Watson-Crick base pairing, thus causing silencing to the target genes. The miRNAs are processed from existing precursor Pri-miRNA, which is processed by Drosha into pre-miRNA in nucleus and exported to cytoplasm and further processed by Dicer to form miRNA, which is subsequently assembled with the Argonaute proteins to form mature RISC for recognizing and targeting RNA by Watson-Crick base pairing, silencing the target genes. This overview for RNAi was extracted from Carthew and Sontheimer (2009)

Recently, domains of nucleotide sequences like CRISPR system have been discovered in the genome of many microorganisms, with the same characteristic structure; short regions of the unique DNA (spacers) are separated from each other by short palindromic repeats. The CRISPR cassettes are in proximity to *cas* genes that encode protein of helicase and nuclease activity (Nemudryi et al. 2014). The spacers DNA of CRISPR loci are derived from DNA of many invading phages and plasmids (Bolotin et al. 2005; Mojica et al. 2005; Pourcel et al. 2005). Interestingly, Cas proteins are encoded by putative operons adjacent to CRISPR sequence; they have functions of nucleases as helicase, polymerase, and RNA-binding proteins (Jansen et al. 2002).

Biogenesis and maturation of CRISPR/Cas system

CRISPR/Cas, an adaptive bacterial immune system to resist the invading selfish genetic elements, was mediated via three stages: adaptation, expression, and interference (Makarova et al. 2011) (Fig. 4). In *adaptation/acquisition phase*, short sequences of ~30 bp homologous to the viral DNA or plasmid are integrated into the CRISPR loci (Barrangou et al. 2007); the acquisition of these spacers are mediated by conserved Cas1 and Cas2 proteins (Brouns et al. 2008). Protospacer selection of foreign invading DNA for integration with bacterial host genome is recognized by protospacer adjacent motif (PAM); this PAM recognition site is different among the various CRISPR/Cas systems (Deveau et al. 2010; Mojica et al. 2005). In *expression phase/crRNA biogenesis*, the pre-crRNA (long transcript of CRISPR locus containing repeats and spacers) are transcribed and processed into mature crRNA, catalyzed by specific endoribonucleases (Makarova et al. 2011). Trans-activating crRNA (tracrRNA) has been recognized as a guide for processing of pre-crRNA in *Streptococcus pyogenes* that are catalyzed by RNase III (Deltcheva et al. 2011). In *interference phase*, the invading DNA or RNA is recognized and cleaved within the active CRISPR/Cas9 system, guided by the crRNA. The mature crRNA and tracrRNA are assembled and complex with Cas9 protein to form active DNA endonuclease (dual-RNA Cas9 system). The crRNA and tracrRNA are combined via terminal tetraloop forming single-guide RNA (sgRNA) (Kim and Kim 2014). CRISPR/Cas9 system has three components: Cas endonuclease, tracrRNA, and crRNA (Jinek et al. 2012) (Fig. 4).

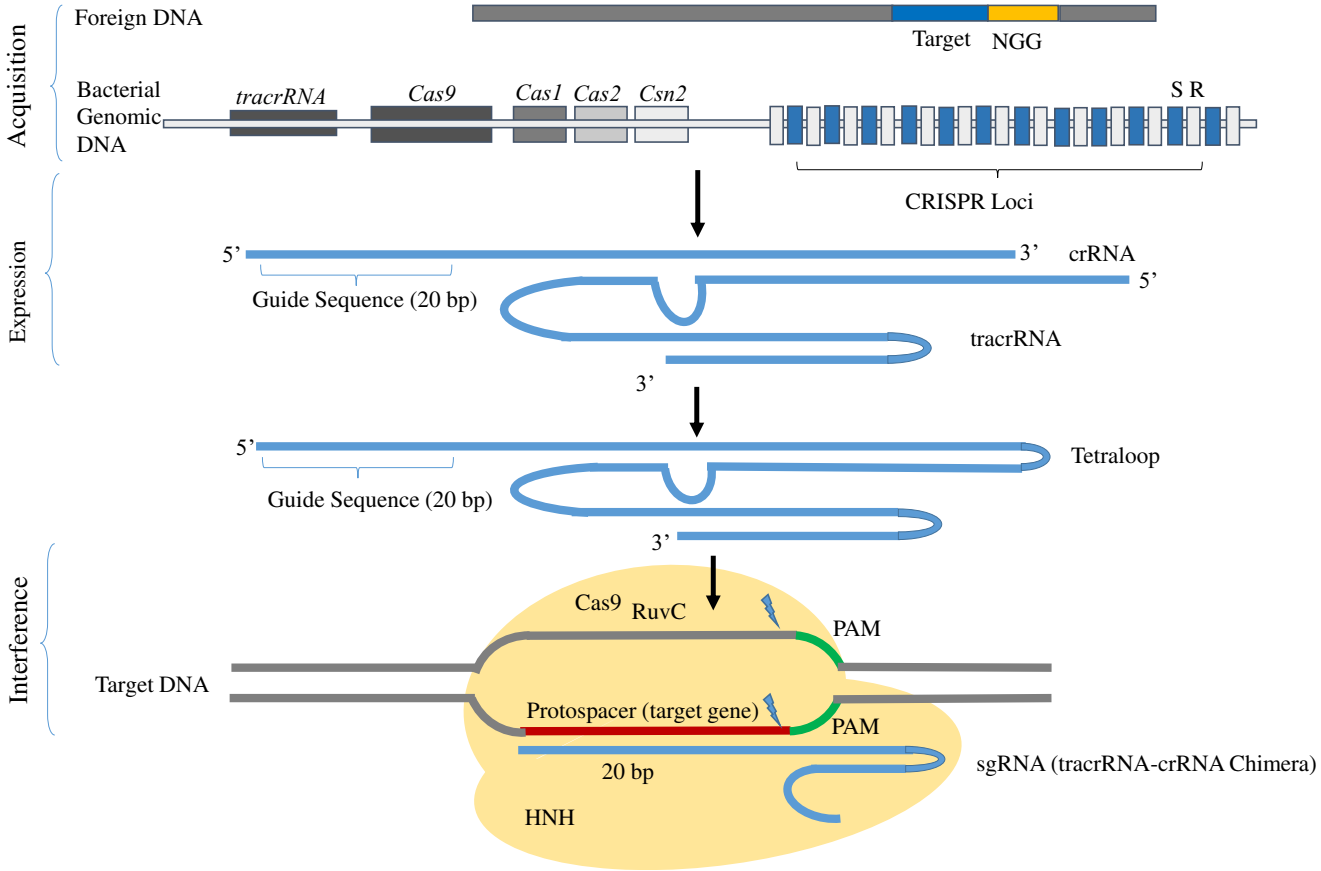

Fig. 4 Schematic representation of CRISPR/Cas9 biogenesis and function. In acquisition phase, the protospacer sequences from the foreign/invading DNA are incorporated into CRISPR loci of bacterial genome, then expression to crRNA and tracrRNA and cas9 protein (expression phase). The four cas genes (cas9, cas1, cas2, and csn2) are located upstream to the CRISPR repeat-spacer arrays consisting of 13 repeats (R) and 12 spacers (S). The tracrRNA is located upstream to cas genes, essential for crRNA maturation. In interference stage, the Cas9 endonuclease complexed with crRNA and tracrRNA, targeting the site-specific sequence of dsDNA containing 20 nt (protospacers) complementary with the designed crRNA adjacent to PAM sites. Fusion of 3′-end of crRNA with the 5′-end of tracrRNA generates chimeric RNA (sgRNA) recognizing the sequence, guiding the Cas9 nuclease cleavage relying on orientation of PAM sites (modified from Jinek et al. 2012)

Thus, combining of crRNA with tracrRNA to one construct short-guide RNA (sgRNA) facilitates the reprogramming of this system for targeting genes, reducing the system components from three into two (Cas9 and sgRNA) (Jinek et al. 2012; Jinek et al. 2014).

In *Staphylococcus epidermidis* and *Sulfolobus solfataricus*, CRISPR system mediates the immunity by resistance to invading DNA of *S. epidermidis* phage and *Sulfolobus* spindle-shaped virus I (SSV1) rather than mRNA (Marraffini and Sontheimer 2008). However, in *P. furoisus*, the CRISPR system targets the foreign invading mRNA (Hale et al. 2009). With the revolution in genomic annotations of CRISPR systems of bacteria, approximately 65 distinct sets of orthologous Cas proteins were discovered, possessing RNase and DNase that categorized into 23–45 families (Haft et al. 2005). Bacterial genomes having CRISPR/Cas system often capture 20 nt of protospacer DNA fragments from the invasive phage or plasmid DNA. The sgRNA (20 nt) of the designed CRISPR/Cas system pairs with the target DNA; then, Cas9 mediates the double-stranded breaks directly upstream to the protospacer adjacent motifs (PAMs) (5′-NGG-3′) by three nucleotides (Hsu et al. 2014; Jiang et al. 2013b; Mali et al. 2013; Ran et al. 2013). The Cas9-DNA complex remains tightly bound to the cleaved DNA with no tendency for multiple cleavage (Sternberg et al. 2014). Strikingly, Cas9 protein from each bacterial genome has a specific recognition PAM sequence (Cong et al. 2013; Jinek et al. 2013; Porteus and Baltimore 2003).

Programmability of CRISPR/Cas9 system

The specificity of Cas9 endonuclease target cleavage is controlled by presence of PAM motifs (5′-NAG-3′, 5′-NGG-3′) downstream to the target sequence that complementary to crRNA. The specificity of CRISPR/Cas9 is further determined by the presence of guanine at 5′-end of the gRNA which is transcribed by RNA polymerase III under control of U6 promotor (Kim and Kim 2014); this limitation was circumvented

using one or two bases at the 5′-end of the sgRNA (Cho et al. 2013; Cho et al. 2014). Imperfect matching of the sgRNA with target sequence is the main cause of off-target cleavage (Bortesi and Fischer 2015). The in silico analyses of eight plants genome sequences revealed that PAM (NGG) sequence was repeated by 5–12 times per every 100 bp of genomic DNA, and concurrently, the number of PAMs are correlated with the genome size (Xie et al. 2015). Currently, it is feasible to designed gRNA to target 68–99% of the plant and microbial genomes. However, with larger genome size, the probability of off-target ratio was noticeably increased; for example, in maize, the targetability of CRISPR is about 30% due to its large genome size (Bortesi and Fischer 2015).

Additionally, the higher mutation rate of gRNA is a limitation for CRISPR/Cas9 system that limits the accessibility of nuclease complex; also, the higher and lower GC contents of gRNA usually have negative effects on functionality of CRISPR (Wang et al. 2014). Cas9 enzyme usually binds to the purine moieties (A, G) of gRNA at the last four residues of spacer sequence; thus, this site has a significant effect on the complexing efficiency of Cas9 and sgRNA (Doench et al. 2014). The mutation rate of CRISPR/Cas9 in plant genome editing is about 13% (Liang et al. 2014); this might be due to the target sequence, gRNA sequence, Cas9 identity, type of cells, and method of delivery (Bortesi and Fischer 2015). For example, protoplasmic transfection of *N. benthamiana* mesophyll by PEG method mediates 37.7% mutation rates, while by agroinfiltration, the mutation rate was 4.8% for PDS gene (Li et al. 2013). The higher frequency of off-target mutations by CRISPR system is one of the main technical limitations of this technology (Cong et al. 2013; Hsu et al. 2014). Among the 20 nucleotides of gRNA sequence, the 8–12 nucleotides at the 3′-end are critical for target site recognition and cleavage that is designated as need sequence (Cong et al. 2013; Jiang et al. 2013b). Any base mismatching along the corresponding gRNA relatively induces the off-target cleavage (Lin et al. 2014). However, the feasibility for reprogramming the CRISPR/Cas9 system to reduce the off-target cleavage by changing the gRNA sequence is the crucial advantage of this system over the ZFN and TALEN technologies (Cho et al. 2014).

Types of CRISPR/Cas system

CRISRPR/Cas systems are categorized into three types based on functionality of Cas proteins and genomic architecture of CRISPR loci (Makarova et al. 2011; Vestergaard et al. 2014). Cas proteins are implemented in diverse stages of CRISPR processing, maturation, and activity. Cas1 and Cas2 proteins are essential for acquisition of the spacer sequences from invading DNA into CRSPR arrays, during the adaptation phase (Nunez et al. 2015). The CRISPR loci were transcribed into pre-crRNA that bound to either Cas9 or multisubunit complex forming crRNA-effector complex (Makarova et al. 2015), subsequently processed into crRNA by endonuclease subunit of the multisubunit effector complex (Wang et al. 2013). The core proteins of different CRISPR/Cas system (Cas 1–10) catalytically have RNase and DNase activities that usually contribute to the CRISPR processing stages (Makarova et al. 2015; van der Oost et al. 2009). The three types of CRISPR/Cas systems (I, II, III) are distinguished by the signature proteins: Cas3 for type I, Cas9 for type II, and Cas10 for type III (Makarova et al. 2015). Recently, another classification of CRISPR/Cas system has been proposed based on the gene clusters encoding the effector modules: class 1 (CRISPR types I, III, and IV) that possess several variants of multisubunit crRNA-effector complexes (CRISPR associated complex for antiviral defense (Cascade), Csm, or Cmr complex) and class 2 (CRISPR types II and V) of Cas9 performing the functions of the effector complex (Makarova et al. 2015). Simply, class 1 CRISPR/Cas system requires complex of Cas proteins for crRNA processing, maturation, and interference, while class 2 requires only single protein (Cas9) for maturation, targeting, and cleavage.

Class 1 CRISPR/Cas system

In *type I CRISPR/Cas system*, it has *cas3* as signature gene encoding helicase and DNase, unwinding the dsDNA and RNA-DNA duplexes (Gong et al. 2014) in addition to encoding genes of Cascade-like complexes (Brouns et al. 2008; Haurwitz et al. 2010). These proteins encompass Cas5, Cas6, and Cas7 based on their sequence analyses (Makarova et al. 2006) as part of repeated-associated mysterious protein (RAMP) superfamily of the Cascade complex. The RAM proteins with RNA endonuclease activity have been recognized to catalyze the transcription of CRISPR long spacer-repeats and processing to mature crRNA (Brouns et al. 2008).

In *type III CRISPR/Cas system*, this system has *cas10* as signature gene encoding multidomain proteins of crRNA-effector complexes mediating the processing and maturation of crRNA for interference stage of CRISPR defense mechanism. The type III loci encode polymerases and RAMP proteins that involved in processing of the spacer-repeat transcripts (Makarova et al. 2006). Type III loci encode small subunit proteins Cas5 and Cas7 as a part of RAMP proteins which are involved in CRISPR transcript processing. The signature Cas10 protein is highly diverse among the different type III CRISPR systems. There are two subtypes of CRISPR III (IIIA and IIIB) that specifically target the DNA and RNA, respectively, in vitro (Rouillon et al. 2013). Class I (CRISPR types I and III) shares the variable RAMP protein that is derivative of RNA recognition motif (RRM) that is often involved in RNA binding and cleavage (Makarova et al. 2015). CRISPR IIIA has *cas1*, *cas2*, and c*as6* genes, while type IIIB loci lack to these genes on bacterial genomes,

ensuring the modularity of CRISPR system (Vestergaard et al. 2014).

In *type IV CRISPR/Cas system*, a functionally uncharacterized putative CRISPR system is found on plasmids of *Acidothiobacillus ferrooxidans* (Makarova et al. 2011), similar to type IIIB CRISPR system, but lacks to *cas1* and *cas2* genes on their array, thus using crRNA from the available different CRISPR arrays. The type IV has *csf1* as signature gene that encodes multisubunit crRNA-effector complex.

Class 2 CRISPR/Cas system

In *type II CRISPR/Cas9 system*, it has *cas9* gene as signature molecule, encoding a single multidomain protein that is essential in generating mature crRNA, cleaving the target DNA, and contributing in spacer sequence acquisition (Heler et al. 2015; Wei et al. 2015). The type II CRISPR/Cas loci have *cas1* and *cas2* genes and tracrRNA, which are complementary to the repeats of corresponding CRISPR array (Briner et al. 2014; Chylinski et al. 2014). Also, type II CRISPR loci encode for a signature *csn2* gene that is crucial for acquisition of invading phage DNA in addition to *cas1* and *cas2* (Heler et al. 2015). In type II CRISPR/Cas9 system, after the adaptation and transcription stages, the interference process initiated with annealing of tracrRNA to the target sequences of pre-crRNA, subsequently cleavage of the dsRNA by host RNase III (Deltcheva et al. 2011). Further processing via trimming of the 5′-end repeat and intermediate crRNA spacer sequence to form mature crRNA was developed (Barrangou and Marraffini 2014). The tracrRNA has two key functions: triggering the processing of pre-crRNA by RNase III and then activating crRNA-guided DNA cleavage of Cas9 (Jinek et al. 2012). In addition to spacer sequence acquisition, Cas1 and Cas2 proteins (metal-dependent endonuclease) are involved in crRNA bio-genesis, targeting the dsDNA (Brouns et al. 2008; Hatoum-Aslan and Marraffini 2014), cleavage into short fragment to serve as spacers, and having DNA integrase/recombinase activity (Wiedenheft et al. 2009). The type II CRISPR/Cas system in *Streptococcus thermophilus* has four *cas* genes (*cas9*, *cas1*, *cas2*, and *csn2*) oriented upstream to 12 repeat-spacer units (Gasiunas et al. 2012).

Cas9 endonuclease structure and function

Cas9 endonuclease is the hallmark protein of type II CRISPR system that is involved in crRNA maturation and crRNA-guided DNA interference (Deltcheva et al. 2011); disruption of *cas9* gene completely abolishes the crRNA-mediated DNA interference (Barrangou et al. 2007). Cas9 proteins are highly abundant among various bacterial genomes with relative variations on sequence and size. Cas9 nuclease has two catalytic domains: HNH domain that cleaves the target DNA strand complementary to the 20 nt cRNA and RuvC domain that cleavage the non-crRNA complementary strand by 3 nt upstream to the PAM site, yielding DSBs (Jinek et al. 2012). This enzyme has a highly conserved arginine-rich region downstream to the region of RuvC domain that is hypothesized to mediate DNA binding (Sampson et al. 2013). Mutation of HNH and RuvC domains modulate the functions of Cas9; mutation of aspartate to alanine (D10A) in RuvC catalytic domain mediates the Cas9 activity to make single-stranded breaks (nickase, Cas9n) rather than DSBs, thus minimize the off-target cleavage (Jinek et al. 2012). The Cas9n (D10A) can use proper sgRNA to nick both strands, mediating DSB of the target DNA locus (Ran et al. 2013). Cas9 genes are clustered into three subfamilies: types II-A, II-B, and II-C (Chylinski et al. 2014). Type A and B subfamilies are about 1400 and 1100 amino acid length; they are being frequently used for genome editing purposes in eukaryotes (Hou et al. 2015).

Streptococcus pyogenes Cas9 (spyCas9) is a type II-A nuclease, with crescent-shaped molecule with 100 × 100 × 50 Å (Jinek et al. 2012). It has bilobed architecture of nuclease lobe containing juxtaposed HNH and RuvC nuclease domains and variable α-helical lobe for DNA binding (recognition lobe) (Jinek et al. 2012). The location of PAM-binding loops on spyCas9 suggests that Cas9 interrogates DNA using flexible regions; there are two tryptophan residues in spyCas9 mediate base-staking interaction with GG dinucleotides of PAM site (Ma et al. 2015). PAM site is critical for directing and guiding the Cas9. The crystal structure of Cas9 in complex with gRNA and target DNA showed that the arginine motifs in its C-terminal domain interact with PAM site of the non-complementary strand in the major groove (Anders et al. 2014). Several orthologous of Cas9 from *Neisseria meningitidis* (Hou et al. 2015), *Streptococcus thermophiles* (Cong et al. 2013), and *Treponema denticola* (Esvelt et al. 2013) have been successfully applied for genome editing approaches.

Three different versions of Cas9 were recognized: (1) wild-type Cas9 that induces DSBs at the target DNA, causing indel mutation, altering the gene sequence, and ultimately gene expression (Heintze et al. 2013); (2) Cas9 D10A nickase (Cas9n) by mutagenesis of aspartic acid to alanine (D10A), generating single-stranded break (SSB) at the target region, thus minimizing the off-target cleavage of the wild Cas9, using a pair of offset sgRNA complementary to the opposite strand of target sites (Cong et al. 2013) (Ran et al. 2013); double nicking of dsDNA by Cas9n causing DSBs can be repaired by NHEJ (error-prone repair), while individual nicks are predominantly repaired by high-fidelity, homology-directed repair (Dianov and Hubscher 2013; Heintze et al. 2013); and (3) dead Cas9 (dCas9) (CRISPRi system). Cas9 catalytically lacks to its endonuclease activity causing gene knockdown/silencing rather than gene knockout. The dCas9 co-expressed with gRNA generates DNA recognition complex that can precisely interfere with the transcriptional regulators, blocking RNA polymerase binding or transcriptional factor binding, and degrading the target mRNA, similar to RNAi silencing

mechanism (Qi et al. 2013). The CRISPRi system has no detectable off-target effects, reversible action, with multiplexing ability for targeting multiple genes simultaneously (Gilbert et al. 2013).

Application of CRISPR/Cas9 system in genome engineering of filamentous fungi

Aspergilli are the most ubiquitous filamentous fungi of enormous pharmaceutical, agricultural, and industrial applications (Ali et al. 2016; El-Sayed et al. 2015a; El-Sayed et al. 2013; El-Sayed et al. 2015b) as well as deleterious effects to humans (Askew 2008). Recently, invasive aspergillosis has dramatically increased with the populations of suspected people, ensuring the urgent need to understand the virulence determinants in these fungi (Upton et al. 2007). The annual invasive aspergillosis is about >200,000 cases due to only infection with *A. fumigatus* > 1.3 million patients have chronic pulmonary aspergillosis and >5 million suffer from allergic broncho-pulmonary aspergillosis (Denning and Bromley 2015). Randomized mutagenesis to identify the virulence genes, pigmentation, and drug resistance has been used (Manavathu et al. 1998); however, unraveling the molecular mechanism of pathogenesis of *A. fumigatus* in vivo and in vitro become an urgent need for targeted drug designing, especially with the emerging of newly antifungal resistant isolates. The proximity of eukaryotic evolutionary relationships of fungi and human makes the traditional antifungals that are quite less effective for their lower selectivity of shared fungal and human metabolism.

Recent genome editing technologies using specific endonuclease offer a new way to understand the virulence genes for selective targeting of the pathogen. Over the past 30 years, CRISPR/Cas9 editing tool had emerged as a powerful editing method in various organisms, however, using this system for genome editing in filamentous fungi still at beginning, despite of the successful applications of this system in yeasts (Jiang et al. 2013a). Few reports of genome editing of *A. fumigatus* to explore its molecular pathogenesis were recently studied with CRISPR/Cas9 system, with >95% in-frame integration (Zhang et al. 2016). The efficiency of this system is about 53% on targeting *A. fumigatus* polyketide synthase gene (*pksP*) generating colorless albino mutant (Fuller et al. 2015), with no deleterious effects of *A. fumigatus* upon expression of Cas9 nuclease. CRISPR/Cas9 system had been used for successful mutagenesis (>80%) of *yA* gene of *A. nidulans* encoding p-diphenol oxidase for green conidial pigmentation, resulting in conidial yellow coloring (Nodvig et al. 2015). Also, this system was used efficiently for mutation of *alba* and *pyrG* genes encoding the black conidial pigmentation of *A. aculeatus* (Nodvig et al. 2015), mutagenesis of *wA*, *yA* (p-diphenol oxidase), and *pyrG* (orotidine 5′-phosphate decarboxylase) genes in *A. oryzae* (Katayama et al. 2016). Also, mutagenesis of *T. reesei URA5* gene that encodes orotidine monophosphate pyrophosphorylase (OMPPase) as negative selectable marker has been proceeded by CRISPR/Cas9 with 100% efficiency (Liu et al. 2015). Inactivation of *ura5* gene was observed by growing *T. reesei* mutant on 5-FOA medium, while the wild-type harboring active gene had no growth due to conversion of 5-FOA into 5′-fluorouridine monophosphate which is selectively toxic for cellular growth. This system was delivered via protoplasmic transformation. Interestingly, expression of CRISPR/Cas9 guided mutagenesis in *T. reesei*. Cas9 expression is controlled by inducing agents as lactose and cellulose and repressed by glucose for *pcbh1* promotor (promotor for *cbh1* gene encoding cellobiohydrolase I) (Liu et al. 2015).

The efficiency and functionality of CRISPR/Cas9 system in filamentous fungi are highly dependent on the transformation system due to the firmness of fungal cell wall that is the main obstacle for successful transformation; thus, protoplast prepared from young hyphae, conidiophore, and spores are reasonable for ideal transformation. Classical PEG-$CaCl_2$ protoplast transformation has been applied in *S. cerevisiae* and *F. oxysporum*, *A. nidulans*, and *N. crassa*. However, liposome-protoplast fusion has been used efficiently for transformation of *Phycomyces blakesleeanus* and *Phytophthora infestans* (Jiang et al. 2013a). Lithium acetate-mediated transformation was used efficiently; Li^+ increases the permeability of plasma membrane, allowing for exogenous DNA penetration (Jiang et al. 2013a). Electroporation and biolistic transformation are more efficient techniques due to increasing the accessibility of target DNA fragment to host cells of protoplast or germinated conidia, applying high-voltage shock to facilitate the uptake of exogenous DNA (Kuo et al. 2004). Biolistic (gene gun) delivery system was shown as reliable tool to transform filamentous fungi, using super-speed gold or tungsten particles to deliver the DNA to the host cells (Djulic et al. 2011). Additionally, *Agrobacterium tumefaciens*-mediated transformation (ATMT) has been successfully used for transformation of various filamentous fungi over the last 15 years. ATMT has been frequently used for gene knockout, knock-in, complementation, and random gene mutagenesis and integration (Jiang et al. 2013a). The evolution in next-generation sequencing for fungi paves the way to exploit a multiple of genome editing tools to manipulate specific "targeted" metabolic pathways. Referring to *Aspergillus* Genome Database (http://www.aspgd.org), sequence and annotation of *A. nidulans*, *A. fumigatus*, *A. flavus*, *A. niger*, and *A. oryzae* genomes had been resolved completely (Cerqueira et al. 2014).

Fungal secondary metabolites

Fungal SMs are diverse natural compounds that emerged from unique de novo biochemical pathways under specific conditions, not directly implemented to normal fungal growth, while it mainly serves to increase the survival fitness of producer, counteracting the competing organisms (Brakhage

2013). Fungal SMs have a tremendous effect on human life, including critical pharmaceutical, industrial, and agricultural relevant compounds, in addition to deleterious compounds (toxins). Fungal SMs are bioactive low weight compounds, restricted to specific life phase, and correlated with sporulation induction, mycelial and conidial pigmentation, and virulence of fungi (Calvo et al. 2002). Genomic studies confirm the presence of SM biosynthetic genes as clusters on fungal chromosomes; however, these genes are silent under standard laboratory conditions or very low expressed, so understanding the biology of these genes is a challenging particularly if the genomes of these fungi are still unknown, in addition to lack of proper genetic tools and lack of sexual system for most of SMs producing fungi (Brakhage 2013). Thus, fungal SM strength and their roles remain enigmatic, since it is regulated by cryptic gene clusters (Brakhage 2013) that are controlled by complex regulatory networks that involve multiple transcriptional factors that respond to environmental stimuli such as nutritional, physical, and interactive signals. Awakening and mining of these cryptic/orphan gene clusters is a challenge for interrogation of fungi for secondary metabolite elicitation. For example, the genome size of *Aspergillus* sp. is about 35 Mb, containing ~50 gene cluster, and only few of them were determined for secondary metabolism, while almost of these clusters are cryptic (Bergmann et al. 2007). Thus, filamentous fungi are considered as untapped reservoir for plethora of natural products, waiting for the future discovery. Microbial communication via co-cultivation was recently elaborated as one of the major signals for activation of fungal cryptic genes cluster that might provide conceptual bioactive metabolites. Co-cultivation of *A. nidulans* with soil-dwelling bacteria had a significant activation on cryptic polyketide synthase (PKS) gene cluster producing orsellinc and lecanoric acid of *A. nidulans* (Abdelmohsen et al. 2015; Ola et al. 2013; Schroeckh et al. 2009). The number of active fungal SMs has been estimated to be more 2000 compounds that were isolated and chemically identified between 1993 and 2005; about half of these compounds had bioactivity as antimicrobial, antitumor, and antimetabolic agents (Keller et al. 2005). There are different classes of fungal metabolites according to their chemical structure and biosynthetic pathways.

Polyketides

Fungal polyketides encompass a large family of diverse bioactive compounds that are synthesized by PKSs. Thousands of polyketide scaffold-derived SMs are clinically relevant compounds as cholesterol-lowering statins and aflatoxins (Hoffmeister and Keller 2007). There are three types of PKSs (I, II, III) based on their modular and domain organization; fungi have iterative type I PKS (Shen 2003). Fungal PKSs are multidomain megasynthase proteins that are similar to fatty acid synthases (FASs) in their structural and functional domains (Keller et al. 2005). Fatty acid and polyketide biosynthetic pathways are started by condensation of acetyl CoA and malonyl CoA forming carbon chains of varying lengths. The metabolic pathway for synthesis of fatty acids and polyketides is shown in Fig. 5. Reduction of β-carbon is the characteristic feature of fatty acid than polyketide synthesis. FAS, an iterative enzyme with multifunctional domains, catalyzes several successive cycles as condensation (β-ketoacyl synthase) of acetyl CoA and malonyl CoA with CO_2 release, forming acetoacetyl-acyl carrier protein (ACP). Subsequently, acetoacetyl-ACP is reduced by β-ketoacyl reductase to form D-3-hydroxybutyrl-ACP, dehydrated by β-ketoacyl dehydratase to crotonyl-ACP, and reduction by enoyl reductase to butyryl-ACP. For elongation, a new malonyl CoA is introduced into acyl-ACP via six successive cycles forming palmitoyl-ACP that is subsequently released from ACP to form palmitate (C16). Fungal FAS is heterododecameric complex (α6 β6) of 2.6-mDa megasynthase. FASs have multidomain proteins such as acetyltransferase (AC), malonyl/palmitoyl transacylase (AT, MPT), β-ketoacyl CoA synthase (KS), β-ketoacyl reductase (KR), β-ketoacyl dehydratase (DH), enoyl reductase (ER), ACP, and thioesterase (TE) that release palmitate from ACP after completion synthesis (Schweizer and Hofmann 2004). AT and MPT are essential for loading the acetyl CoA and malonyl-CoA to ACP (Jenni et al. 2007; Lynen 1980).

Statin biosynthesis in fungi Statins are the major group of polyketides that are used as cholesterol-lowering agents by inhibiting β-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase, key enzyme in mevalonate pathway for cholesterol biosynthesis. Completely synthetic statins as atorvastatin (Lipitor), semisynthetic as simvastatin (Zocor), pravastatin (pravacol), and naturally lovastatin and compactin (Weng et al. 2010) were the committed drugs for hypercholesterolemia. Lovastatin (mevinolin, mevacor, monacolin K) and compactin were initially produced from *A. terreus*, *Monoascus ruber*, and *Penicillium citrinum* in addition to other fungal isolates (Hendrickson et al. 1999; Mulder et al. 2015). Lovastatin has been approved by FDA in 2002 and revolutionized as antihypercholesterolemic drug with preventative efficacy in treatment of atherosclerosis, sepsis, and arterial and vascular diseases (Alberts 1988). Genetically, lovastatin has been encoded by cluster of 18 genes in *A. terreus*; five genes of this cluster were nominated for synthesis of this metabolite, namely, *lovA*, *lovB*, *lovC*, *lovD*, and *lovF* (Kennedy et al. 1999) (Fig. 6). The genes *lovB* and *lovF* encode the core of PKSs, while *lovC*, *lovD*, and *lovA* encode ER, TE, and cytochrome P450 oxygenase, respectively, and *lovE* and *lovH* as transcriptional factors (Kennedy et al. 1999). The *lovB* and *lovF* are large multidomain megasynthases that are homologous in tertiary structure and domain orientation and synergistically encode polyketides. The *lovB* encodes lovastatin nonaketide synthase (LNKS; EC. 2.3.1.161) of 335 kDa that is highly iterative type I PKS with the catalytic

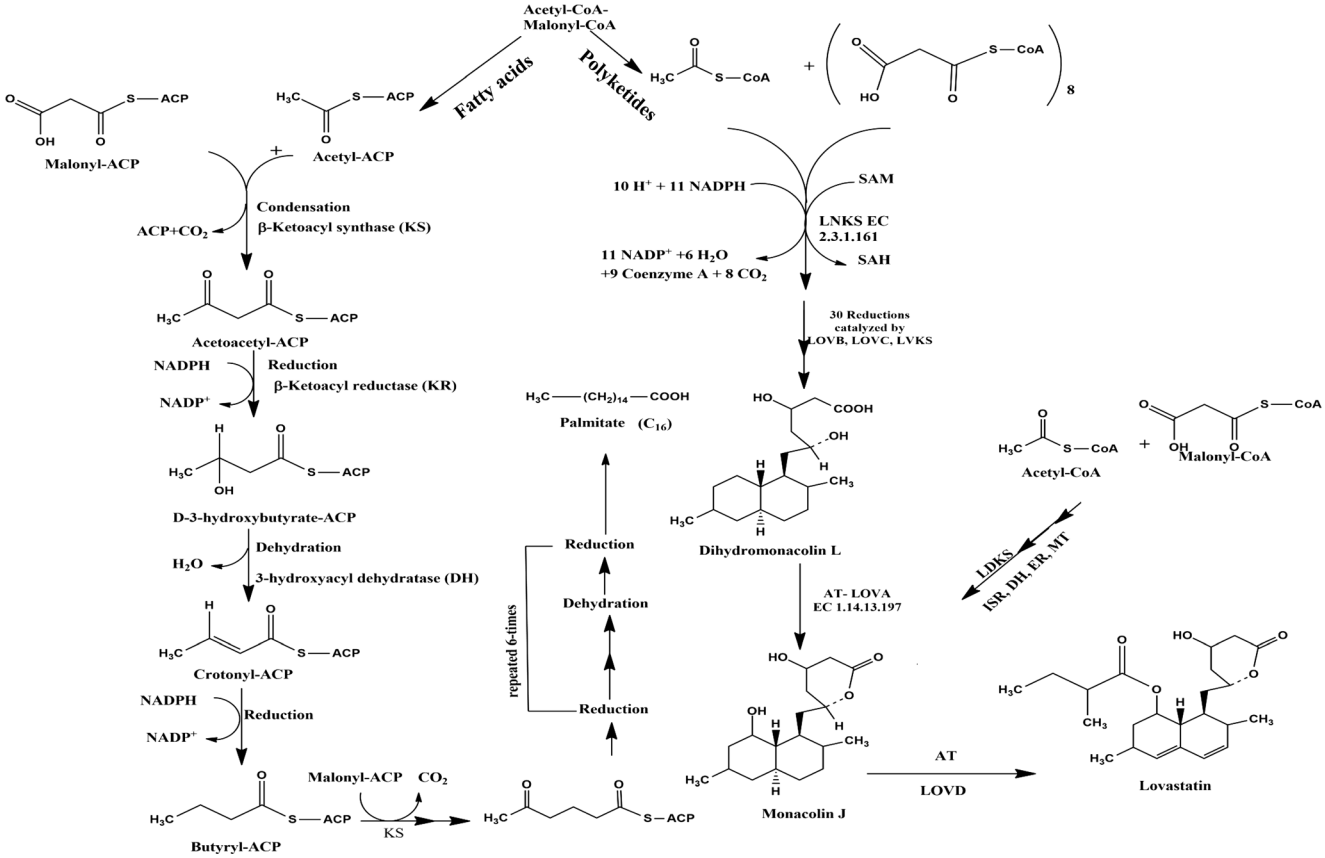

Fig. 5 Polyketide and fatty acid biosyntheses in filamentous fungi. Acetyl CoA is the main precursors for polyketides and fatty acids syntheses; the two units of acetyl CoA and malonyl CoA are loaded to acyl carrier protein to form acetyl-ACP and malonyl-ACP that subsequently condensed through the catalytic domains of FAS and PKS to synthesis of fatty acids and lovastatin

domains KS, AT, malonyl transferase (MT), DH, methyltransferase (MT), KR, and ACP (Fig. 6) (Kennedy et al. 1999). Enoyl reductase domain is inactive on *lovB*; thus, LNKS requires *lovC* as separate domain. LNKS catalyzes the iterative initial key stage of lovastatin synthesis, building the main nonaketide core, while the *lovC* constructs the 2-methylbutyryl side chain (Ames et al. 2012). The coupled *lovB* and *lovC* genes are synchronized to catalyze 30 reactions precisely yielding 19-carbon intermediate dihydromonacolin L. Lovastatin biosynthesis started with condensation of 8 malonyl-CoA, 1 acetyl Co-A, 11 NADPH, and 1 *S*-adenosylmethionine (SAM) molecule to form dihydromonacolin L (Bizukojc and Ledakowicz 2009). Once the acetyl unit binds to *lovB*, a Claisen condensation that proceed was catalyzed by KS domain followed by KR till nonaketide formation, followed by DH domain activity to form heptaketide. The MT domain catalyzes the transfer of methyl group from

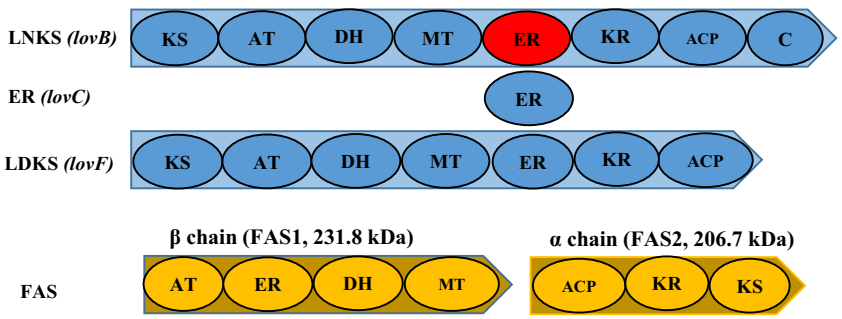

Fig. 6 Fungal polyketides synthase (PKS) and fatty acid synthase (FAS) domain structure. The catalytic domains of PKS and FAS are β-ketosynthase (KS), acetyl transferase (AT), dehydratase (DH), methyltransferase (MT), enoyl reductase (ER), ketoreductase (KR), acyl carrier protein (ACP), and cyclase (C). The DH, MT, ER, and ER of lovB (*red*) domains are inactive in PKS, while they are active in FAS. FAS two subunits are β and α

SAM to grow tetraketide, followed by Diels-Alder cycloadditions to hexaketide to form dihydromonacolin L (Kelly 2008) (Fig. 6). The *lovF* gene that encodes lovastatin diketide synthase (LDKS) of 277 kDa has the same domains of LNKS with active ER domain that catalyzes one Claisen condensation to produce 2-methylbutyryl, subsequently attached to hydroxyl group of C8 of monacolin J to produce lovastatin (Ames et al. 2012).

PKSs and FASs are structurally similar, multimodular/multidomain megaenzymes; however, the PKSs lack the reduction or dehydration reactions that give more diversity, in addition to post-PKS modification, providing a wide range of bioactive compounds (Crawford and Townsend 2010). Mutagenesis of lovastatin biosynthetic gene cluster (*lov* genes) of *A. terreus* using cerulenin as specific PKS inhibitor completely blocked lovastatin biosynthetic pathways, directing the fungal metabolism to produce oktaketide and emodin. Overexpressions of *lovB*, *lovD*, and *lovF* had a strong improvement on lovastatin biosynthesis. Recently, engineering of the side chain of monacolin J for production of semivastatin use was developed (Xie and Tang 2007). *lovG* encoding thioesterase was identified as the essential enzyme for dihydromonacolin L releases from LNKS regulating the LNKS turnover for continued synthesis of lovastatin (Xu et al. 2013).

Non-ribosomal peptides

A group of FSMs having proteinogenic and non-proteinogenic amino acids were encoded by non-ribosomal peptide synthetases (NRPS). NRPS is a multimodular enzyme; each module have several domains for adenylation (A), pantothenylation/peptidyl carrier (P), condensation/peptide bond formation (C), and TE domains (Finking and Marahiel 2004). These domains are essential for recognition, activation, and covalent binding of module-specific amino acids as thioester to the 4′-phosphopantetheine cofactor of each module (Keller et al. 2005). Penicillin and cephalosporin were the first NRPs recognized from *Penicillium chrysogenum* that were encoded by NRPS gene cluster as δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase (ACVS) (Smith et al. 1990) and isopenicillin N synthase (Keller et al. 2005). Siderophore ferricrocin as NRP of cyclic hexapeptide residues encoded by NRPS was recognized from *A. nidulans* (Keller et al. 2005).

Terpenes

Terpenoids are the most diverse microbial SMs; over 40,000 different terpenoids have been isolated and characterized from microbes. The common fungal terpenoids are taxol, carotenoids, gibberellins, trichothecenes, and sterols that were recognized with their strong bioactivity (Roberts 2007). Terpenoid biosyntheses are derived from the starting units: isopentenyl diphosphate (IPP) and its allylic isomer dimethyl allyl diphosphate (DMAPP) (isoprene units, C5). Different classes of terpenoids based on the building block isoprene units (C5) and monoterpenes are generated from condensation of IPP and DMAPP to form geranyl diphosphate (GPP; C10) by GPP synthase, sesquiterpenes which are generated from farnesyl diphosphate (FPP; C15) through condensation of one isoprene unit to GPP, and diterpenoids that are derived from geranylgeranyl pyrophosphate (GGPP; C20) by GGPP synthetase. Higher terpenoids as sterols (C30) are derived from triterpenoid squalene, from condensation of six isoprene units, or by condensation of two FPP molecules. Carotenoids (C40) are derived from condensation of two molecules of GGPP, eight units of isoprene. Thus, GPP, FPP, and GGPP are the main building blocks for various terpenoids via terpene cyclase with additional oxidation, reduction, and isomerization (Roberts 2007). The properties and crystal structure of terpene cyclase, essential for isoprene unit condensation, have been resolved from *Fusarium*, *Aspergillus*, and *Penicillium* spp. (Keller et al. 2005).

Taxol biosynthesis in fungi

Taxol is one of the most clinically valuable highly oxygenated terpenoids since its discovery in 1970 from the Pacific yew tree, *Taxus brevifolia* (Wani et al. 1971). Taxol has been recognized as blockbuster anticancer compound, the most successful broad range drug against proliferation of numerous cancers such as breast and ovarian and AIDS-related sarcoma; it has been approved by FDA in 1994 (Croteau et al. 2006). Taxol has a unique mode of action by binding to β-tubulin, promoting the microtubule assembly, and disrupting the mitotic division of the target cells (Sharma et al. 1993). Taxol biosynthesis involves more than 20 steps from progenitor diterpenoid geranylgeranyl diphosphate that was recognized firstly in genus *Taxus* (Croteau et al. 2006). The committed biosynthetic pathway of taxol started from the isoprene units; IPP and DMAPP derived from mevalonate and isoprenoid pathways are shown in Fig. 7. Mevalonate pathway started by the synthesis of 3-hydroxy-3-methylglutaryl-CoA (HMG)-CoA from condensation of acetyl-CoA and acetoacetyl-CoA by HMG-CoA synthase (HMGS), subsequently reduced by HMG-CoA reductase to mevalonic acid (Chappell et al. 1995). HMG-CoA reductase is a highly regulated, rate-limiting enzyme of mevalonate pathway (Goldstein and Brown 1990). Mevalonate is phosphorylated by mevalonate kinase to phosphomevalonate that is further phosphorylated by phosphomevalonate kinase (PMK) to mevalonate pyrophosphate, then decarboxylated by mevalonate pyrophosphate decarboxylase (MVPD) to IPP. Furthermore, IPP is isomerized by action of IPP isomerase to dimethyl allyl pyrophosphate (DMAPP). The IPP and DMAPP isoprene unites (C5) are condensed to GGP (C10), the basic structural unit of monoterpenes (C10) such as geraniol,

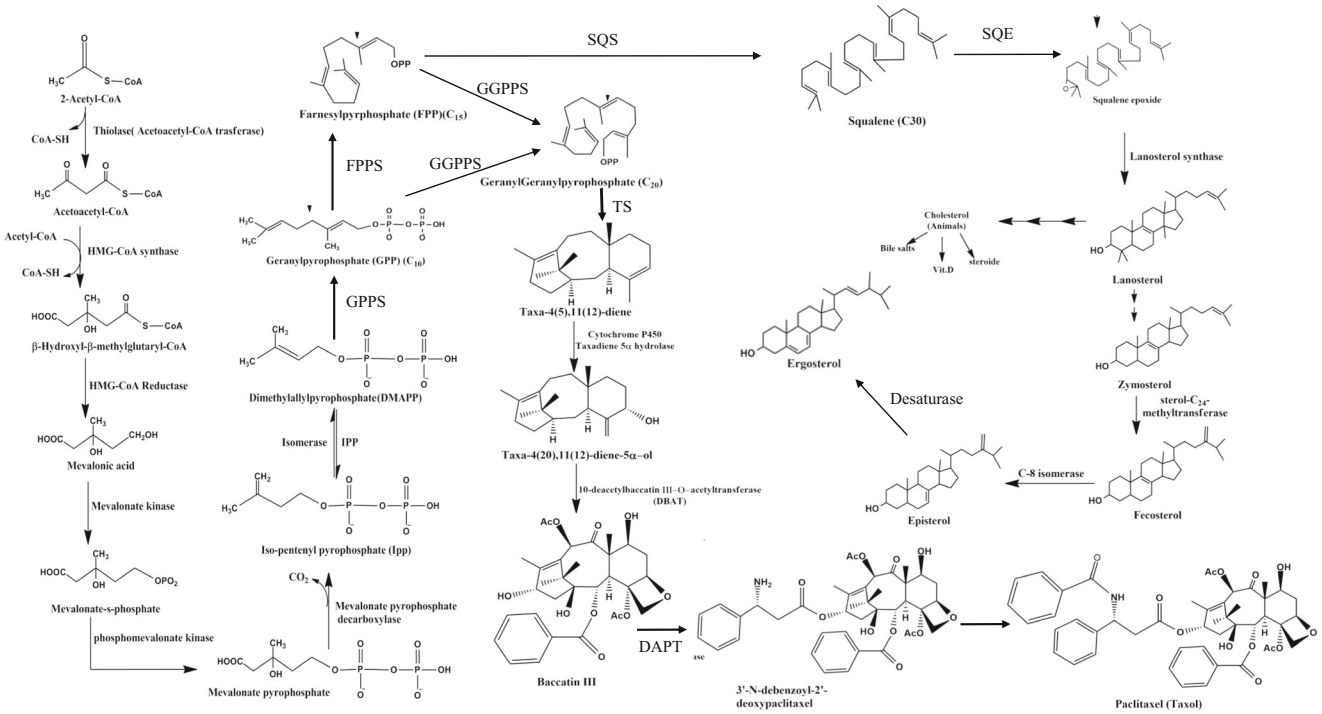

Fig. 7 Biosynthesis of terpenoids in fungi. Mevalonate and isoprenoids pathways are the main sources of isopentenyl pyrophosphate (IPP) and dimethyl allyl pyrophosphate (DMAPP) (isoprene units, C5). Farnesyl pyrophosphate (FPP) is the precursors of diterpenoids (taxol, C20) and sterols (C30) through condensation reactions catalyzed by geranylgeranyl pyrophosphate synthase and squalene synthase, respectively. *GPPS* geranyl pyrophosphate synthase, *FPPS* farnesyl pyrophosphate synthase, *GGPPS* geranyl geranyl pyrophosphate synthase, *SQS* squalene synthase, *SQE* squalene epoxidase, *TS* taxa-4(5),11(12)-diene synthase, *DAPT* baccatin III −13-O-(3-phenylpyropanyoyl) transferase (DAPT), *DBTNBT* 3′-N-benzoyl-2′-deoxytaxol-N-benzoyltransferase

limonene, linalool, and citronella, by geranyl pyrophosphate synthase (Ogura and Koyama 1998; Wriessnegger and Pichler 2013) (Fig. 7). FPP (C15) is formed by condensation of GPP (C10) and IPP (C5) by farnesyl pyrophosphate synthase (FPPS); FPP units are the precursors for sesquiterpenes (C15) (Wriessnegger and Pichler 2013). By geranylgeranyl pyrophosphate synthase (GGPPS), isoprene unit IPP (C5) and FPP (C15) or two units of GPP (C10) were condensed to form GGPP diterpenoid (C20) that is the precursor for taxadiene, via cyclization and oxygenation with substitution of several functional groups via 19 steps of chemical reactions (Jennewein et al. 2004b).

Taxol biosynthesis has been committed by the following steps (Fig. 7): (1) formation of taxadiene via cyclization of GGPP by action of taxadiene synthase (TS), a monomeric protein of 75 kDa; (2) oxygenation by addition of seven functional oxygen at multiple sites of taxadiene by cytochrome P450 monooxygenase, then hydroxylation of C5 position of taxane ring by cytochrome P450 taxa-diene-5α-hydroxylase (T5αH) forming taxa-4(20),11(12)-dien-5α-ol (Jennewein et al. 2004a); (3) oxidation of ketone, formation of oxetane ring, and side chain assembly that were catalyzed by taxadiene-5α-ol-*O*-acetyl transferase and 10-deacetylbaccatin III-O-acetyltransferase (DBAT) to baccatin III; and (4) hydroxylation of 3′-N-debenzoyl-2′-deoxypaclitaxel side chains to paclitaxel (taxol) by 3′-N-debenzoyl-2′-deoxtaxol-N-benzoyltranserase (DBTNBT) (Kusari et al. 2014; Malik et al. 2011; Roberts 2007).

Taxol production and endophytic fungi

Taxol uses have been expanded for treatment of metastatic ovarian carcinoma, breast cancer, and non-small-cell lung cancer and second-line treatment of AIDS-related Kaposi's sarcoma in addition to recent trials against Alzheimer's and Parkinsonism (Zhang et al. 2005). Overall, diterpenoid extracted from natural source is often in insufficient yield, usually correlated with unpredicted fluctuation in the environmental conditions of the producer. The taxol concentration is about 0.001–0.05% in *T. brevifolia*, which is the most productive species. Thus, for production of 1 g of taxol, it needs about 10 kg of *Taxus* bark (~60-year age), which is produced approximately from three trees, and every cancer patient requires about 2.5 g (Malik et al. 2011). Owing to the growing demand for taxol and shortage of mature tree of *T. bervifolia*, searching for alternative technologies like chemical synthesis, plant cell culture, microbial fermentations of endophytes, and metabolic engineering of microorganisms raise the hope for industrial production of this drug (Wriessnegger and Pichler 2013). The total global market of taxol is above $ 1 billion per year, with more predictable grossing with anticipation trials of

this drug in various diseases (Ganem and Franke 2007). Tissue culture technology achieves a successful development for paclitaxel production, but the long incubation time is still one of the main limiting factors with the urgent accessibility of cancer patients worldwide.

Fungal endophytes from different *Taxus* spp. were reported to have a strong correlation with taxol productivity, plant growth promoters, and other secondary metabolites (Kusari et al. 2013). Taxol biosynthesis by *Taxus* sp. was significantly increased by coculturing with *Fusarium mairei* or its purified oligosaccharides (Li et al. 2009) that was explained by the microbial cross talking due to activation of some cryptic genes. Endophyte taxol producer *Paraconiothyrium* sp. induced the expression of taxol biosynthetic gene in *Taxus* sp. that makes the plant more resistant to specific pathogens; strikingly, the fungus has a potential selectivity to inhibit the host plant pathogenic fungi. Taxol-biosynthetic genes in *Paraconiothyrium* sp. were induced (eightfold higher) by both non-taxol-producing endophytes as *Alternaria* sp. and *Phomopsis* from the same host plant, *Taxus* (Soliman and Raizada 2013). Notably, all the endophytes producing taxol are naturally resistant to taxol which has been shown as a powerful fungicide to plethora of phytopathogenic fungi (Suryanarayanan et al. 2009). *Taxomyces andreanae* was the first isolated taxol-producing endophyte from *Taxus* spp., raising the hope for production of taxol via fermentation process, because of its fast growth on simple cultural media, possibility for growing on bulk fermenters, resistance to shearing, and feasibility for genetic manipulation (Stierle et al. 1993). Acetate and phenylalanine were the precursors of fungal taxol, and its yield was dramatically increased in presence of sterol biosynthesis inhibitors as tebuconazole and triadimefon (LI et al. 1998). Several fungal endophytes belonging to *Ascomycetes* and *Deutromycetes* have been recognized as promising paclitaxel producers, among these genera *Pestalotia*, *Pestalotiopsis*, *Sporomia*, *Trichothecium*, *Tubercularia*, *Alternaria*, *Pithomyces*, *Monochaetia*, *Penicillium*, and *Fusarium* (Flores-Bustamante et al. 2010). Interestingly, 150 fungal isolates were identified as endophytes from *T. baccata*; more than 10% of this population have the ability to produce taxol such as *Alternaria*, *Beauveria*, *Epicoccum*, *Fusarium*, *Geotrichum*, *Phoma*, *Nodulasporium*, *Phomopsis*, and *Fusarium solani* (Caruso et al. 2000). TS has been used as molecular marker for screening of taxol-producing fungi from *T. chinensis*. Among the 38 endophytic fungi, 12 isolates were reported as TS positive, but only 3 isolates gave a detectable yield of taxol. Additionally, 10-deacetylbaccatin III-10-O-acetyl transferase (DBAT) and phenylpropanoyl side chain CoA acetyltransferase (BAPT) are more diagnostic markers for taxol biosynthesis (Zhang et al. 2009).

Microbial engineering is the reliable platform for large-scale production of terpenoids in a cost-effective fermentation process, independent on climate changes and cultivation risks. Several metabolic approaches have been implemented based on increasing the influx of IPP via mevalonate pathway (Siddiqui et al. 2012). Taxol biosynthesis is controlled by 19 enzymatic steps that are part of the terpenoid pathway; thus, several trials were conducted to increase the yield of taxol via overexpression of these enzymes in yeast or *Escherichia coli.* Overexpression of pyruvate dehydrogenase, acetaldehyde dehydrogenase, and acetyl-CoA synthetase for increasing the supply for acetyl-CoA has a significant positive effect on the yield of terpenoids synthesis in *S. cerevisiae* (Shiba et al. 2007). Recently, engineering of mevalonate production by introducing mevalonate decarboxylase "mevalonate bypass," reducing the total number of ATP from three to one and the number of enzyme from seven in original pathway into four, has a strong great effect on the taxol yield (Kang et al. 2016). Overexpression of taxadiene synthase, HMG-CoA synthase and reductase, and GGPP synthase in *S. cerevisiae* has positively effect on the taxadiene yield that was increased by about 40-fold (Engels et al. 2008). Due to the complexity of enzymatic system for terpenoid synthesis, there are multiple methods for enhancing the yield of taxol such as blocking/silencing the competitive pathway, overexpression of the key genes, and increasing the feeding precursors (Roberts 2007). Metabolic engineering of microbial cells to enhance the flux of precursor pools of IPP and DMAPP via overexpression of mevalonate and isoprenoid pathway rate-limiting enzymes has been recognized as a reliable tool for enhancing the yield of terpenoids.

Sterol biosynthesis in fungi

Sterols are group of terpenoids that are found in eukaryotic cell membranes, essential for growth, maintaining appropriate membrane fluidity, and regulating membrane-bound enzymes, membrane permeability, and functionality (Joffrion and Cushion 2010). Fungal and mammalian cell membranes have ergosterol and cholesterol, respectively, while plant cell membranes have complex sterols as sitosterol, stigmasterol, and campesterol (Volkman 2003). The committed enzymatic systems for sterol biosynthesis in mammals and fungi are mostly conserved; these enzymes are initiated based on FPP (C15), from condensation of two isoprene units (IPP and DMAPP) that are derived from isoprenoid pathway (Olivier and Krisans 2000). FPP is the basic unit for sterol, ubiquinone, dolichols, heme A, and prenylated proteins in human. Condensation of two molecules of FPP gives pre-squalene diphosphate (PSDP), followed by NADH-dependent reductive rearrangement to produce squalene, catalyzed by squalene synthase (SQS; EC. 2.5.1.21) (Lee and Poulter 2008). HMG-Co-reductase and SQS were used as powerful targets for blocking the sterol biosynthesis. Subsequently, squalene epoxidase oxidizes squalene to squalene epoxide, the first tetracyclic sterol skeleton (C30) that was further cyclized by

catalysis of lanosterol synthase to lanosterol, the initial precursor for sterol synthesis in fungi and mammals. Demethylation of C14 lanosterol ring is performed via two stages of oxidative reactions catalyzed by lanosterol-14α-methyl demethylase (C14α-demethylase) generating unsaturated sterol, 4,4 dimethyl-cholesta 8,14,24-trienol, followed by reduction of C14 to 4,4-dimethylzymo-sterol then to zymosterol (Joffrion and Cushion 2010). In fungi, zymosterol is further metabolized by C24-methyltransferase (EC. 2.1.1.41) to form fecosterol (Aoyama et al. 1984). Methylation of C24 of zymosterol is the most determinant step for ergosterol biosynthesis in fungi that does not exist in cholesterol synthesis in mammalian cells. SAM is the source of methyl group to methylate C24 of zymosterol to produce episterol that catalyzed SAM-Δ^{24-}sterol methyltransferase (SMT; EC. 2.1.1.42) (Pereira et al. 2010). This enzyme is absent in mammalian sterol synthesis, that it could be a selective target for antifungal drugs.

The enzymatic systems of sterol synthesis are conserved among fungi and mammals till lanosterol synthesis (Joffrion and Cushion 2010). In fungal cell membrane, ergosterol has a monomer layer arrangement, making it more rigid with less flexibility, unlike the bilayer arrangement of cholesterol in mammalian cell membranes. Mammalian cells have no cell walls, and the bilayer arrangement of cholesterol makes their plasma membranes more flexible for more protection than fungal membranes. In fungi, the unsaturation of ergosterol by C5 and C22 desaturases is correlated with the rigidity of their cell membranes (Hildenbrand and Bayerl 2005). However, exceptionally to the presence of ergosterol in fungi, some opportunistic fungi *Pneumonia carinii* are unable to synthesize their sterol and thus scavenger cholesterol from the immunocompromised host, thus attaining a resistance to antifungal drugs targeting ergosterol (Joffrion and Cushion 2010).

Prospective manipulation of lovastatin and taxol biosynthesis using CRISPR/Cas9

Recently, gene manipulation has received great attention with the tremendous advances on system biology and synthetic biology with more feasibility for multiplex gene engineering for a purposeful modification of cellular metabolism. In contrary to random mutagenesis, metabolic engineering allows precise cellular modification, avoiding unnecessary cellular metabolites. Several metabolic engineering strategies have been succeeded to redirect the microbial metabolic flux towards the desired metabolite such as (1) increasing supply of precursors, (2) overexpressing the bottleneck enzymes, (3) transcriptional regulations of gene expression, (4) tunable control of the metabolic pathway, (5) enzyme engineering, (6) deletion/minimizing the flux for unsolicited pathways/competing pathways, (7) activation of the cryptic genes, and (8) promotor engineering (O'Connor 2015; Pickens et al. 2011).

Regarding the continuous global need to lovastatin and taxol as influential anticholesterol and anticancer drugs, as mentioned previously, economically, the marketing of these drugs has been estimated by hundreds of $ billions in 2015. Thus, addressing the probable metabolic engineering pathways for enhancing the yield of these active compounds using CRISPR/Cas9 technology was the objective of this review. Currently, CRISPR/Cas9 system tool does a revolution in genome editing of filamentous fungi, especially *Aspergillus* spp. (Zhang et al. 2016; Nodvig et al. 2015; Katayama et al. 2016). The overall metabolic biosynthetic pathways for lovastatin and taxol in fungi and the hypothetical engineering strategies to manipulate the rate-limiting enzymes to enhance the yield of these metabolite are illustrated in Fig. 8. Metabolite manipulation via overexpression of bottleneck enzymes and knockout of competing pathways controlling genes are the most powerful tools to establish new metabolomic pathways, increasing the flux towards the desired metabolite and switch-off the underside metabolic flux (Lee et al. 2009).

Lovastatin biosynthesis In terms of increasing the precursor supply, overexpression of acetyl and malonyl transferases could have a strong positive effect to the flux of these pathways to produce polyketides and fatty acids, thus limiting the flux of these starter and extender primers from the terpenoid biosynthesis as competing metabolic pathway. Amelioration of the precursors supply has positive impact on primary and secondary metabolites. Carboxylation of acetyl-CoA to malonyl-CoA by acetyl CoA-carboxylase (ACC) is a rate-limiting step for polyketide and fatty acid syntheses. Overexpression of ACC has increased the polyketide yield by about sixfold in *Streptomyces coelicolor* (Ryu et al. 2006). And overexpression of ACC and acetyl-CoA synthetase and blocking the malonyl-CoA degradative pathways has increased the intracellular yield of malonyl-CoA by 15-fold (Zha et al. 2009). Nevertheless, supplementation of the metabolism of cells with the precursors supply may enforce the cellular metabolism to primary metabolites rather than secondary metabolites, so engineering of the pathway-specific components, via overexpression of its rate-limiting enzyme controlling lovastatin synthesis, could be the alternative pathway. Gene overexpression/dosing has been used successfully for multiplication of penicillin by about 64-fold, via increasing the copies of the NRPS gene cluster in *P. chrysogenum* (Smith et al. 1989). Consistently, overexpression of specific domains of *lovB*-encoding LNKS and *lovC* (enoyl reductase) as the main rate-limiting step enzymes in lovastatin synthesis shed lights about the remarkable substrate discrimination necessary for dihydromonacolin L synthesis. Enoyl reductase has been shown to catalyze three of eight possible polyketide synthesis stages, namely, tetra, penta, and heptaketide stages of

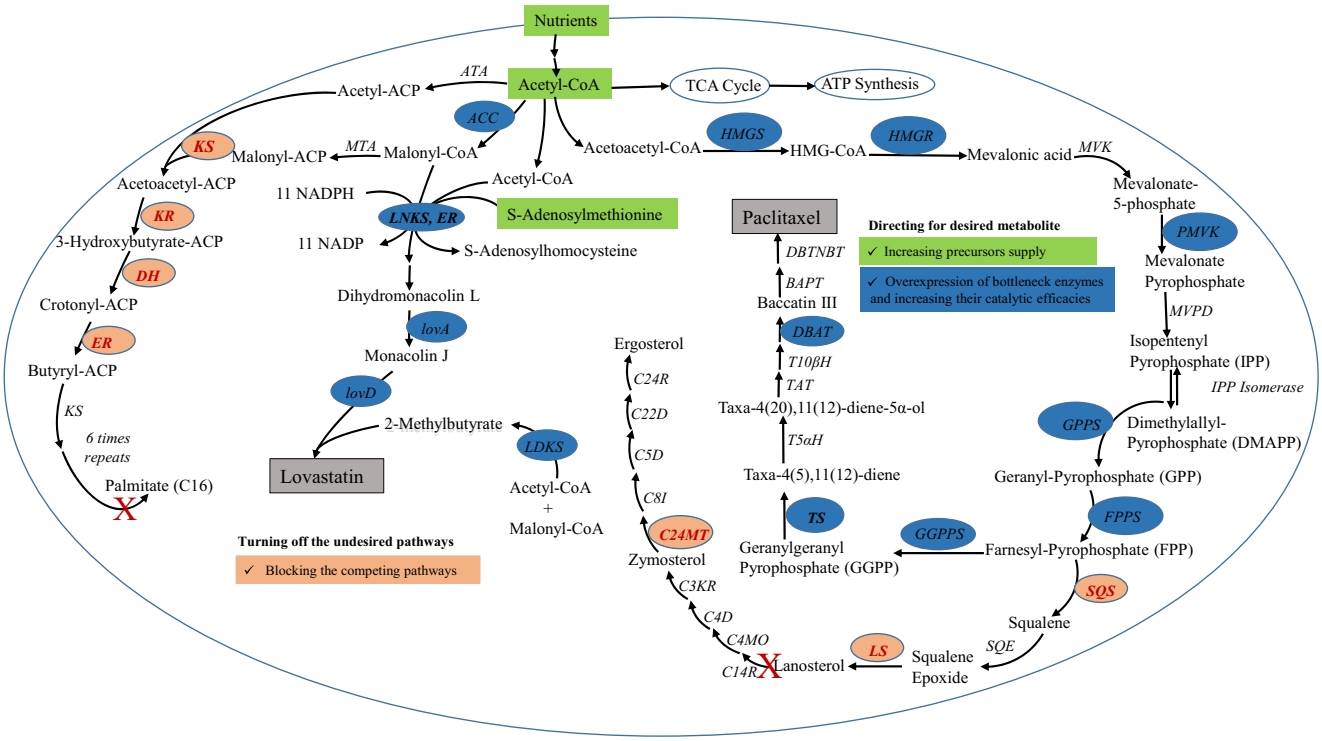

Fig. 8 Overall metabolic biosynthetic pathways of lovastatin and taxol in fungi and the prospective engineering strategies to manipulate the rate-limiting enzymes and enhance the yield of these metabolites. The proposed three metabolic engineering protocols for enhancing the lovastatin and taxol yield from fungi were increasing the precursors supply (*green box*), overexpression of the rate-limiting enzymes (*blue box*), and blocking of the competing pathways (*yellow box*). Overexpression of the key enzymes ACC, LNKS, ER, and LDKS for lovastatin synthesis and GGPPPS, TS, and DBAT for taxol synthesis (*blue color*) are the current metabolic engineering approaches. Blocking of the competing metabolic pathways by knocking out the rate-limiting enzymes for fatty acid synthesis and sterol synthesis to enhance the yield of lovastatin and taxol is the promising metabolic engineering prospects for CRISPR/Cas9 technology applications

dihydromonacolin L synthesis which are still physically bind to LNKS. Thus, overexpression of ER could have a potential for overall lovastatin synthesis (Kennedy et al. 1999). Enoyl reductase has trans-acting activity in lovastatin. This feature has been reported among other fungal PKSs as general strategy for diverse polyketide. However, the specificity in lovastatin synthesis is still ambiguous. Also, LDKS encoded by *lovF* is essential for synthesis of side chain 2-methylbutyryl of lovastatin, as confirmed from the mutant of *lovF A. terreus*, that accumulated monacolin J with no lovastatin (Kennedy et al. 1999). Moreover, in association of *lovF*, *lovD* gene (encoding transesterase) is essential for binding of 2-methylbutyryl to *lovF* and monacolin J, forming lovastatin, since *lovF* lacks to TE domain. Thus, exploitation of CRISPR/Cas9 technology for precise overexpressions of *lovB*, *lovC*, *lovD*, and *lovF* could pave the way for new insights for maximizing the yield of lovastatin or its derivatives. With the advantages of gene dosing/increasing gene copy number, control of the gene expression for lovastatin biosynthesis seems to be talented technique to fine-tune metabolic engineering. Using of inducible promotor system to simultaneously regulate the expression of upstream and downstream modules of lovastatin could be a novel strategy for its yield optimization (Pickens et al. 2011).

In addition, overexpression of the rate-limiting enzymes and expression of inducible promotors, deleting of the competing pathways, are one of the most successful metabolic engineering avenues. The competing pathways usually siphon off the precursors from the desired metabolic pathway and contribute to unsolicited metabolites; thus, blocking of these pathways could be dramatically beneficial. Biochemically, lovastatin biosynthesis (polyketide) competes with the terpenoids and fatty biosynthetic pathways (Fig. 8). Sterol is the major terpene biosynthesized by fungi via mevalonate and isoprenoid metabolic pathways. Thus, blocking the mevalonate pathway by knocking out of rate-limiting enzyme HMG-CoA reductase could emphasize the lovastatin synthesis. Thus, CRISPR/Cas9 system for knocking out of HMG-Co reductase could ensure the blocking of mevalonate pathway, thus reducing the downstream metabolites for sterol and other terpenoid syntheses. Similar results that were documented by knocking out the endogenous squalene synthase *erg9* gene resulted in blocking the downstream metabolites and redirecting of flux to amorphadiene biosynthesis in *S. cerevisiae* (Paradise et al. 2008), and knocking out of hydrolase had a dramatic increase on simvastatin yield (Xie et al. 2007). The chemical inhibitors for sterol synthesis were committed such as bisphosphonates and zaragozic acids to specifically inhibit FPPS and squalene synthase, thus blocking the sterol downstream

biosynthesis (Buhaescu and Izzedine 2007). The commitment of these chemical inhibitors to block the flux sterol synthesis offers a real opportunity for exploitation of genome editing CRISPR/Cas9, thus avoiding the unwanted chemical interactions and probability of stopping desired pathways. Association of (+)-geodin derived from anthraquinone emodin with lovastatin in the culture filtrate of *A. terreus* is one of the challenges for lovastatin production. From the transcriptomic analysis, dihydrogeodin oxidase was recognized as a key enzyme in (+)-geodin biosynthesis, and this enzyme is structurally correlated to the enzymes controlling pigment biosynthesis. Knocking out of dihydrogeodin oxidase had a significant effect on reduction of (+)-geodin biosynthesis compared to control (Askenazi et al. 2003). Thus, disruption of the putative biosynthetic pathways of (+)-geodin by CRISPR/Cas9 system could offer a novel tool for blocking this competing pathway, enhancing lovastatin yield.

Fatty acid and polyketide syntheses are very competing metabolic pathways, since the structural domains of PKSs and FASs are very similar; thus, redirecting the metabolite flux to lovastatin synthesis via blocking the fatty acid synthesis is a challenge for the structural proximity of PKS and FAS domains (Fig. 6). However, the domains of KR and DH are mainly required for ketone reduction in fatty acid synthesis, but not active in some PKSs for lovastatin synthesis (Keller et al. 2005). The structural domains of fungal FAS and their modulation for substrate shuttling had been extensively studied (Jenni et al. 2007). Thus, modulation or knocking out of these two structural domains (KR and DH) by CRISPR/Cas9 system could have a positive effect on directing acetyl-CoA to the main flux of statin biosynthesis.

Taxol biosynthesis Metabolic engineering of endophytic fungi for overproduction of taxol is one of the most biotechnological aspects since the discovery of *T. andreanae* as a potent taxol producer under in vitro axenic culture conditions (Stierle et al. 1993). In 993, this discovery raises the hope of biotechnologists to bulk production of this drug to be commercially more accessible; however, the inability of this fungus to give the desired reproducible yield of taxol in addition to gradual loss of the potency for production with continuous subculturing was disappointing to continue for industrial scale-up (Kusari et al. 2014). Currently, more than 300 endophytic fungi mostly from *Ascomycetes* and *Deutromycetes* have been included to the list of taxol producers, but unfortunately, none of these fungi have been translated into industrial scale. In addition to lower yield of taxol by endophytic fungi, the drastic loss to its production with repeated subculturing, with deficiency to comprehensive understanding to their biology and biochemistry, prevents the devoted use of these endophytes as host plant-independent taxol producers (Heinig et al. 2013). The loss of taxol productivity with repeated endophyte subculturing might be explained on the basis of losing, inactivation of some crucial genes with culture aging due to reprogramming of fungal physiology, and regulatory circuit. The biosynthetic pathway of taxol in *Taxus* spp. implementing about 20 enzymatic steps has been well characterized; however, full molecular signature and anabolism of taxol from fungal endophytes have not been resolved yet. Arguments about the temporarily harboring of taxol biosynthetic genes from *Taxus* host plants to the endophytes have been negated, since some endophytes isolated from non-taxol producing plants have taxol-producing potency (Kusari et al. 2014). The process of horizontal gene transfers (HGTs) might have some contribution in transferring of the taxol-biosynthetic genes from taxol-producing microbes to non-taxol producer endophytes.

The molecular biosynthetic mechanism of taxol from endophytes is not fully understood; however, the basic isoprene units (IPP, BMAPP) as building blocks for GGPP biosynthesis from mevalonate and isoprenoid pathways are well characterized. Thus, increasing the precursors (IPP, DMAPP) by overexpression of the rate-limiting HMG-Co synthase and HMG-Co reductase could have dramatic positive effect on terpenoid production. Overexpression of HMG-CoA reductase in yeasts, while knocking out of squalene synthase, resulted in enrichment of the cells with endogenous FPP for terpenoid synthesis (Martin et al. 2003). Since the overexpression of mevalonate and isoprenoid synthetic pathway might be contributed to extra primary metabolites and unwanted metabolic pathways, overexpressions of taxadiene synthase and *BAPT* as a rate-limiting enzyme were succeeded for improving the taxol yield. Overexpression of TS gene to endophytic fungi under the control of specific promotors leads to dramatic increases in taxol yield (Engels et al. 2008). In combination with the overexpression of taxol biosynthetic genes, blocking the competing downstream metabolic pathways is being a reasonable strategy. Since the different diterpenoids and sterol synthesis are competing for farnesyl pyrophosphate, thus knocking out of squalene synthase/lanosterol synthase, rate-limiting enzymes in sterol synthesis could strongly direct the metabolic flux to taxol biosynthesis (Do et al. 2009). Squalene synthase inhibitors have been recognized to strongly inhibit the sterol yield, enhancing the other branch "taxol" anabolism (Davidson 2007). Thus, by blocking sterol biosynthesis, the GGPP pool units were directed towards taxol synthesis. Metabolic directing of fungal metabolism to taxol biosynthesis was detected using specific sterol synthesis inhibitors. For example, incorporation of taxol production media of *P. microspora* with tebuconzole and triadimefon increased the yield of taxol by sixfold compared to control (LI et al. 1998). From the literatures, blocking the sterol biosynthetic pathway, as strong competitive pathway for taxol synthesis, has a dramatically positive effect on taxol yield. Thus, exploitation of CRISPR/Cas9 system for knocking out the squalene

synthase and lanosterol synthase could be the prospective tool for bulk industrial production of taxol.

Concluding remarks and future directions

With the evolution of genomics and metabolomics, filamentous fungi have been recognized as untapped reservoir for bioactive secondary metabolites; theoretically, each fungus has more than 30 PKSs oriented on secondary metabolic gene clusters. Thus, with combinatorial synthetic and experimental studies, marvelous secondary metabolites of medical values remain to be discovered from fungi. Lovastatin and taxol and their chemical derivatives have been recognized as the most global marketed drugs for treatment of hypercholesterolemia and cancer diseases. However, the lower natural yields of these compounds from their native producing fungi and consequently their lower accessibilities into industrial scale are the main technological hurdles. Chemical attempts for scaffold optimization and structural modifications are quite challengeable and laborious, requiring multiple chemical reactions. Thus, metabolic engineering of fungi for overproduction of these compounds and their chemical derivatives has been recognized as powerful technology for integration of the laboratory pilot scale to industry scale. Traditional methods as optimizing the cultivation/nutritional conditions to elicit the implemented cryptic genes of secondary metabolite synthesis has been extensively documented, but still does not support the anticipated yield. Using chemical inhibitors to block specific downstream metabolic pathways to direct the intermediate metabolites to synthesis of taxol and lovastatin had a reliable progress on increasing the yield of these compounds. Unlike the limited efficiency of traditional methods, molecular metabolic engineering could be a more powerful in time saving, feasibility, and yield of the target compounds. Molecular metabolic engineering via overexpression of the precursors supply controlling enzymes and rate-limiting enzymes for biosynthesis of these compounds has been sophisticated by the high-throughput omics and computational analysis (Park et al. 2008). Moreover, with revolution on high-throughput omic techniques and bioinformatics, metabolic engineering via transcription regulators, promotor engineering, rate-limiting enzyme engineering, tunable control of metabolic pathways, and blocking the competing pathways could significantly contribute in improving the fungal productivity to these compounds.

CRISPR/Cas9 system has emerged as a powerful and precise tool of genome engineering of various organisms; it has shown a remarkable success in genome editing of filamentous fungi. Exploitation of CRISPR/Cas9 technology for knocking out the downstream metabolism mediating enzymes as HMG-Co reductase, squalene synthase, fatty acid dehydratase, and reductase domains, as rate-limiting enzymes in sterol and fatty acid syntheses, could have a dramatic positive effect on the yield of lovastatin. Similarly, blocking the sterol metabolic pathway by knocking out the squalene synthase and lanosterol synthase by CRISPR/Cas9 system genome editing tool could greatly enhance the yield of paclitaxel. Till date, it is premature to proclaim that the CRISPR/Cas9 technology is in a golden age on metabolic engineering of fungi for pharmaceutical products. Nonetheless, exploitation of CRISPR/Cas9 genome editing technology coupled with activation of the cryptic secondary metabolites gene clusters represents one of the most promising avenues for manipulation of fungi for drugs and agrochemical discoveries.

Compliance with ethical standards The manuscript has no studies with humans or animals.

Conflict of interest The authors declare that they have no conflict of interest.

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