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MINI-REVIEW

Insights into *Monascus* biology at the genetic level

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Abstract The genus of *Monascus* was nominated by van Tieghem in 1884, but its fermented product—red mold rice (RMR), namely red yeast rice, has been used as folk medicines, food colorants, and fermentation starters for more than thousands of years in oriental countries. Nowadays, RMR is widely developed as food supplements around the world due to its functional compounds such as monacolin K (MK, also called lovastatin) and γ-aminobutyric acid. But the usage of RMR also incurs controversy resulting from contamination of citrinin (a kind of mycotoxin) produced by some *Monascus* strains. In the past decade, it has made great progress to *Monascus* spp. at the genetic level with the application of molecular biology techniques to restrain the citrinin production and increase the yields of MK and pigment in RMR, as well as aid *Monascus* classification and phylogenesis. Up to now, hundreds of papers about *Monascus* molecular biology (MMB) have been published in the international primary journals. However, to our knowledge, there is no MMB review issued until now. In this review, current understanding of *Monascus* spp. from the view of molecular biology will be covered and insights into research areas that need to be further investigated will also be discussed.

Keywords *Monascus* spp. · DNA markers · Secondary metabolism · G protein signaling pathway · *Monascus* genome

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Introduction

The genus of *Monascus*, belonging to the family Monascaceae, the order Eurotiales, the subclass Eurotiomycetidae, and the class Eurotiomycetes (Geiser et al. 2006), was nominated by van Tieghem in 1884. In many oriental countries, *Monascus* strains are inoculated to steamed rice to produce red mold rice (RMR), which is also called Hongqu, red yeast rice, Hon-Chi, Anka, red koji, red Chinese rice, and red fermented rice—a kind of traditional fermentation food and a traditional Chinese medicine for curing indigestion, dysentery, and relieving pain (Li and Guo 2003; Lin et al. 2008). It has been proved that RMR contains many kinds of beneficial compounds, such as edible pigments (widely used as the natural food colorant), monacolins (a group of anti-hypercholesterolemic agent), gamma-amino butyric acid (GABA, a kind of hypotensive agent), dimerumic acid (a natural antioxidant), sterols, isoflavones, unsaturated fatty acids, protease, and esterase (Lin et al. 2008; Shi and Pan 2011; Patakova 2013). In recent years, many scientific researches demonstrated that RMR had potential therapeutic action to several chronic diseases, even to prevention of cancer development (Liu et al. 2003; Arunachalam and Narmadhapriya 2011; Lee and Pan 2011; Hsu and Pan 2012; Lee and Pan 2012, 2013; Shi and Pan 2012; Hsu et al. 2013), so RMR has been utilized as a popular dietary supplement worldwide. However, the discovery of citrinin (a kind of mycotoxin) production by *Monascus* strains aroused controversy over the safety of RMR (Blanc et al. 1995a; Wang et al.

2005). Now, different countries show different attitudes to RMR and related products. For instances, effort to use *Monascus* pigments as food colorants in Europe and the USA was ceased, while RMR is permitted successive usage as food additives in Asian countries such as China with the maximum residue level (Zhou et al. 2012; Patakova 2013).

In recent years, several genes involved in the biosynthesis of citrinin, monacolin K (MK), and pigments and G protein signaling pathway (Shimizu et al. 2005, 2007; Chen et al. 2008a, 2010; Yang et al. 2012; Balakrishnan et al. 2013; Li et al. 2010a, 2014) have been cloned and analyzed, which made an important step forward in understanding the secondary metabolism in *Monascus* spp.. Although hundreds of papers about *Monascus* molecular biology (MMB) have been published in primary research journals, there is still no MMB review issued up to now. To help audience understand the recent research in rapidly progressing and emerging areas of MMB, available knowledge on *Monascus* spp., including molecular classification and phylogenesis, gene clusters of secondary metabolites, related genes to G protein signal transduction pathway, and genomic information, will be covered in this review, and insights into research areas that need further to be investigated will also be discussed, enhancing our understanding of *Monascus* spp. in the view of fungal biology.

Molecular classification and phylogenesis of *Monascus* spp.

Monascus spp., a kind of filamentous fungi, isolated from RMR, was classified and named by van Tieghem in 1884. Since the genus of *Monascus* was recognized into three species including *Monascus ruber*, *Monascus pilosus*, and *Monascus purpureus* in 1983 (Hawksworth and Pitt), more than 20 species, which are listed in Table 1, have been recorded in documentary materials (Li and Guo 2003; Park and Jong 2003). In fact, only nine *Monascus* species have been widely accepted. The existing disparity may be attributed to the synonyms of *Monascus* spp.. Therefore, some molecular markers such as random amplified polymorphic DNA (RAPD), D1/D2 regions of large subunit ribosomal RNA (LSU rRNA), internal transcribed spacer (ITS) and partial β-tubulin genes, *Monascus* retrotransposon (MRT) as well as inter-simple sequence repeat (ISSR) and sequence-related amplified polymorphism (SRAP) have been used to assist the *Monascus* classification and phylogenesis, which are free from the effects of variation of cultural conditions on morphological and physiological characteristics, providing alternative tools for discrimination of *Monascus* spp. (Park et al. 2004).

RAPD marker was firstly used to study the genetic relationship within 25 isolates of *Monascus*, and cluster analysis indicated these isolates represented four genetic lineages of *Monascus*, suggesting that a relatively restricted genetic source of *Monascus* isolates is used in food products in Asian countries (Lakrod et al. 2000). Shinzato et al. (2009) also demonstrated that RAPD was available to differentiate closely related *Monascus* strains. Based on the sequence information of D1/D2 regions of LSU rRNA genes, 65 strains of *Monascus* and *Xeromyces* were clustered into five groups with *Aspergillus anthodesmis*, *Penicillium inflatum*, *Cephalotheca sulfurea*, *Albertiniella polyporicola*, and *Aporothielavia leptoderma* as outgroup control (Park and Jong 2003). Later, 17 ATCC strains of *Monascus* species were differentiated according to the sequence comparison of ITS and partial β-tubulin genes (Park et al. 2004). These investigations showed that the combination of morphological analysis and sequence comparison can not only differentiate *Monascus* species, but also confirm the synonyms of some species. It is noteworthy that the phylogenetic analysis based on β-tubulin gene is consistent with alignments postulated by DNA sequences of the D1/D2 region of LSU gene, but phylogenetic analysis with ITS sequence information should be cautiously used due to the insertion and deletion of DNA sequence (Park et al. 2004). Chen et al. (2007) inferred that the species of *Monascus* can be grouped by the presence or absence of MRT elements via Southern hybridization based on phylogenetic subgroups established with the partial β-tubulin gene, but this method is a time-consuming process. In addition, some DNA markers are developed to study genetic diversity, classification, and identification of species according to the structural feature of genomic DNA, such as inter-simple sequence repeat (ISSR) marker and sequence-related amplified polymorphism (SRAP) technique. In 2011, the genetic diversities of 37 *Monascus* isolates from different samples gathered in China, and 14 reference strains from the microbial collections in the world, were analyzed by ISSR and SRAP. According to the dendrogram produced by two sets of data, 51 tested strains were grouped into four clusters at the 70 % similarity level, sharing great similarity with each other except minor differences in the subgroups. And the analysis was in agreement with morphological observations, revealing that both SRAP and ISSR have potential to discriminate the *Monascus* strains (Shao et al. 2011).

In short, it goes to show that these DNA molecular markers will be alternative approaches to investigate the polymorphism of *Monascus* spp. in both interspecies and intraspecies. But different molecular markers have their own applicable scopes based on different experiment principles and target DNA region.

Functional investigation on interest of genes (gene clusters) of *Monascus* spp.

In the contemporary era, a number of investigations have proven that besides *Monascus* pigments, *Monascus* spp.

Table 1 *Monascus* species recorded in different documents since 1983 to now

Strains recorded in documents	Reference	Annotations
Monascus ruber Van Tieghem	Hawksworth and Pitt (1983)	Acceptable internationally
M. pilosus K. Sato ex D. Hawkswoth and Pitt	Hawksworth and Pitt (1983)	Acceptable internationally
M. purpureus Went	Hawksworth and Pitt (1983)	Acceptable internationally
M. floridanus Cannon and Barnard	Barnard and Cannon (1987)	Acceptable internationally
M. pallens Canon, Abdullah and Abbas	Cannon et al. (1995)	Acceptable internationally
M. sanguineus Canon. Abdullah and Abbas	Cannon et al. (1995)	Acceptable internationally
M. eremophilus Hocking and Pitt	Hocking and Pitt (1988)	Acceptable internationally
M. lunisporas Udagawa and Baba	Udagawa and Baba (1998)	Acceptable internationally
M. argentinensis	Stchigel et al.(2004)	Acceptable internationally
M. anka Nakasawa et and Sato	Li and Guo(2003); Park and Jong (2003)	
M .anka var. *rubellus* Sato	Li and Guo (2003); Park and Jong (2003)	
M. albidus var. *glaber* Sato	Li and Guo (2003); Park and Jong (2003)	
M. kaoliang Tsai, Hseu and Shen	Li and Guo (2003); Park and Jong (2003)	
M. fuliginosus Sato	Li and Guo (2003); Park and Jong (2003)	
M. albidus Sato	Li and Guo (2003); Park and Jong (2003)	
M. araneosus Sato	Li and Guo (2003); Park and Jong (2003)	
M. barkeri Dangeard	Li and Guo (2003); Park and Jong (2003)	
M. bisporus (Fraser) v. Arx	Li and Guo (2003)	
M. aurantiacus Li	Li (1982)	
M. serorubescens Sato	Li and Guo (2003); Park and Jong (2003)	
M. vitreus Sato	Li and Guo (2003); Park and Jong (2003)	
Monascus rubropunctatus Sato	Li and Guo(2003); Park and Jong(2003)	
Monascus major Sato	Li and Guo (2003); Park and Jong (2003)	
M. paxii Lingelsheim	Li and Guo (2003); Park and Jong (2003)	
M. pubigerus sato	Li and Guo (2003)	
M. olei Pidallu	Li and Guo (2003)	
M. rubiginosus Sato	Li and Guo (2003); Park and Jong (2003)	
M. heterosporus (Harz) Schroter	Li and Guo (2003)	
M. mucorroides van Tieghem	Li and Guo (2003)	
M. vini Savulescu and Hulea	Li and Guo (2003)	

can produce many other bioactive compounds including monacolins, GABA, dimerumic acid, and so on, and also citrinin, a kind of mycotoxin (Aniya et al. 1999; Blanc et al. 1995a; Su et al. 2003; Patakova 2013). So, as to efficiently increase the amount of functional materials but decrease the citrinin contents, it becomes a research hotspot to illuminate the biosynthetic pathways of the metabolites in *Monascus* spp.. With the introduction of molecular genetic tool into *Monascus* research, the genes involved in the biosynthesis of several main metabolites from *Monascus* spp. have been or are being investigated. In the following sections, the available transformation methods, gene (cluster) involving in the synthesis of citrinin, MK and pigment in *Monascus* spp. will be described in detail.

DNA transformation processes of *Monascus* spp.

In the past decade, molecular mechanisms involving in metabolism and development of filamentous fungi have made great progress since the first genetic manipulation of *Neurospora crassa* was reported by Case et al. (1979). However, there was little genetic knowledge of *Monascus* due to lack of suitable molecular genetic tools until the late of the twentieth century. Therefore, it is necessary to develop efficient transformation methods to introduce exogenous DNA sequence into the genomic DNA of *Monascus*. Since the first genetic transformation of *Monascus* spp. was reported via genomic DNA-mediated transformation (GDMT) in 2003 (Lakrod et al. 2003a), biolistic bombardment (Lakrod et al. 2003b), polyethylene glycol-mediated protoplast

transformation (PEG-MPT), *Agrobacterium tumefaciens*-mediated transformation (ATMT) (Campoy et al. 2003), and restricted-enzyme mediated integration (REMI) technique also have been successfully applied to *Monascus* genetic transformation (Zhou et al. 2006; Chen et al. 2008b). Compared with the transformation methods described above, it can be found that the transformants obtained by GDMT were always mitotically unstable (Lakrod et al. 2003a), and transformation efficiency was lower with biolistic bombardment or PEG-MPT (Lakrod et al. 2003b; Zhang et al. 2013), while REMI technique was a time-consuming process dependent on the selection of restricted-enzyme (Zhou et al. 2006; Chen et al. 2008a). Instead of the aforementioned shortages, ATMT, with its appealing features, such as no protoplast preparation, higher transformation efficiency, a simpler manipulation process, and more stable DNA integration, may be the most suitable molecular transformation method to *Monascus* spp. until now (Michielse et al. 2005). The transformation methods applied to *Monascus* spp. in recent years are listed in Table 2.

Gene clusters of secondary metabolites in *Monascus* spp.

Filamentous fungi can produce diverse secondary metabolites mainly including polyketides (PKs), non-ribosomal peptides (NRPs), terpenes (TPs), and indole terpenes (ITPs) (Staunton and Weissman 2001; Fox and Howlett 2008; Brakhagea and Schroeckha 2011; Wiemann et al. 2013). The biosynthetic genes responsible for these compounds are usually clustered together, which have been proved by many researches (Fox and Howlett 2008; Brakhagea and Schroeckha 2011; Brakhage 2013). In *Monascus* spp., citrinin, pigment, and MK are typical PKs compounds, which were catalyzed by polyketide synthases (PKSs) consisting of a minimal set of ketosynthase (KS), acyltransferase (AT), and acyl carrier protein (ACP) domains (Staunton and Weissman 2001; Lackner et al. 2012). According to this structural feature, several PKSs in *Monascus* spp. were cloned and analyzed functionally, which will be described in the following sections.

Citrinin gene cluster

Citrinin was firstly discovered as an antibacterial factor named monascidin A in RMR (Wong and Koehler 1981). Later, Blanc et al. (1995a, b) confirmed that monascidin A was the same compound as citrinin, a kind of mycotoxin, which had been identified as a secondary metabolite in a variety of fungi such as *Aspergillus* spp. and *Penicillium* spp.(Sweeney and Dobson 1998; Malmstrøm et al. 2000). For preventing the contamination of *Monascus*-fermented products from citrinin, many scientists have been focusing on the breeding and optimization of fermentation conditions to reduce citrinin in RMR (Chen and Hu 2005; Lee et al. 2007; Pattanagul et al. 2008; Hajjaj et al. 2012), but it is very difficult to be absolutely eliminated. Hence, many current researches focus on the isolation of the gene cluster involved in citrinin biosynthesis and elucidation of the bio-catalyzed process in *Monascus* spp..

In 2005, *pksCT*, a PKS gene (GenBank accession no. AB167465) in *M. purpureus* was first isolated with degenerate primers targeting the conserved KS domain of a fungal PKS, which had a length of 7,838 bp with a single 56-bp intron, and encoded a 2,593-amino acid protein that contained putative domains for KS, AT, ACP, and a methyltransferase (Shimizu et al. 2005). Following these information, a *pksCT*-inactivated mutant without the KS domain or the ACP domain was constructed, which did not produce citrinin, also had no influence on pigment production, demonstrating that *pksCT* was directly responsible for the citrinin biosynthesis in *M. purpureus*. However, the resulting *pksCT* disruptant was unstable and could become its revertant by repeat cultivation (Shimizu et al. 2005). Based on the cloned DNA sequences, a 21-kbp fragment (GenBank accession no. AB243687) was cloned including four open reading frames (ORFs) (*orf1*, *orf2*, *orf3*, and *orf4*) in the 5′-flanking region and one ORF (*orf5*) in the 3′-flanking region in the vicinity of *pksCT* from *M. purpureus*, and a major activator (*ctnA*) of citrinin biosynthesis was also identified by site-specific recombination (Shimizu et al. 2007). Sequence analysis revealed that *ctnA* gene encoded a protein sharing significant similarity to the members of the Zn(II)2Cys6 binuclear DNA binding proteins. The disruption of *ctnA* caused large decreases in the transcription of *pksCT* and orf5 (the putative membrane transporter), as well as significant reduction of citrinin production, while complementation of the *ctnA* disruptant restored the transcription of citrinin-related gene and its production. Fu et al. (2007) also constructed a *pksCT*-deleted mutant of *Monascus aurantiacus*, resulting to a 97.2 % decrease of citrinin amount, while the production of red and yellow pigments increased to 49.4 and 28.8 %, respectively. Later, Li et al. (2012) isolated a larger DNA fragment with 43-kb length from *M. aurantiacus* by probes hybridization with a fosmid library, which was deduced as 16 ORFs including the homologous gene of *pksCT*, 7 ORFs (*ctnD*, *ctnE*, *orf6*, *orf1*, *ctnA*, *orf3*, and *orf4*) in the 5′-flanking region and 8 ORFs in the 3′-flanking of *pksCT* (*orf5*, *ctnF*, *orf7*, *ctnR*, *orf8*, *ctnG*, *ctnH*, and *ctnI*). Then, they deleted the *orf4* (*ctnB*, encoding for a predicted oxidoreductase) through homologous recombination and the results revealed that Δ*ctnB* mutants barely produced citrinin, suggesting that the *ctnB* gene is directly involved in citrinin biosynthesis. Based on the plausible citrinin biosynthetic gene cluster cloned by Shimizu et al. (2005), Sakai et al. (2008) introduced an additional copy of activator gene (*ctnA*) together with the gene cluster into *Aspergillus oryzae*, and developed a heterologous expression system to produce citrinin. In order to decrease the contamination of citrinin, several genes in citrinin biosynthetic cluster in *Monascus* spp. were disrupted to create citrinin-free or citrinin-

Table 2 The transformation methods applied to *Monascus* spp. in recent years

Recipient	TM	SM	TE	Reference
Protoplast of albino *M. purpureus*	Electroporation	Color expression	$5/10^9$ Protoplast recipient	Lakrod et al. (2003a)
Protoplast of albino *M. purpureus*	PEG-mediated transformation	Phleomycin	$43/1.8\sim3.0\times10^8$ Protoplasts	Campoy et al. (2003)
Spores of *M. purpureus*	ATMT	Hygromycin	$123\pm12/2\times10^7$ Protoplasts	Campoy et al. (2003)
Protoplast of *M. purpureus* DSM1379	Electroporation	Hygromycin	$4\times10^2/10^8$ Protoplasts.	Kim et al. (2003)
Conidia of albino *M. purpureus*	Biolistic transformation	Hygromycin	1/10 μg DNA donor	Lakrod et al. (2003b)
spores of *M. ruber*	ATMT	Hygromycin	35/plate	Shao et al. (2006)
Protoplast *of M. purpureus*	Protoplast-PEG method	Aureobasidin A	17 Colonies/ug DNA	Shimizu et al.(2006)
Conidia of *M. ruber*	ATMT	Hygromycin	$800–1{,}000/10^6$	Yang and Lee (2008)
Spores of *M. purpureus*	ATMT	Hygromycin	26/plate	Cai et al. (2010)
Conidia of *M. albidus* 9901	ATMT	Hygromycin	52/plate	Wang et al. (2011)

TM transformation methods; *SM* selectable markers; *TE* transformation efficiency

depressed mutants, providing a potential approach to genetically modify the *Monascus* strains (Jia et al. 2010; Li et al. 2013).

MK gene cluster

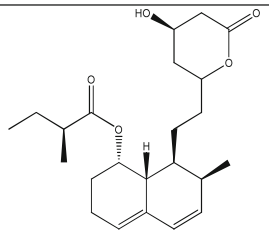
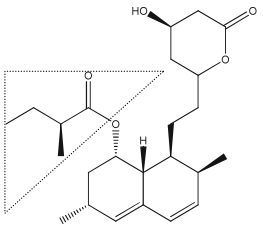
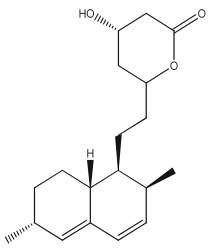
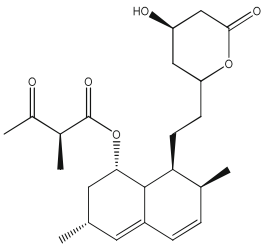
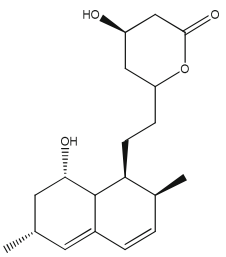
In the 1970s, Japanese scholar Ando discovered MK in the fermentation broth of *M. ruber*, the same substance as lovastatin and compactin produced by *Aspergillus terreus* and *Penicillium citrinum*, respectively (their chemical structures were listed in Table 3), which can significantly reduce serum cholesterol level by competitively inhibiting the activity of 3-hydroxy-3-methyl glutaryl coenzyme A (HMG-CoA) reductase (Endo 1979). Afterwards, Ando further isolated several MK analogs from *Monascus* spp. including monacolin L, X, J, M, dihydroerinolin and dihydromonalin L, and all of them possessed the same basic structure as MK and strong cholesterol-lowering effect (Endo et al. 1985, 1986). Nowadays, MK has become one of the most secure drugs for the reduction of cholesterol with best application effect, attracting extensive attentions from China and abroad (Grabley and Thiericke 1999).

As what was mentioned before, some strains from both *Aspergillus* spp. and *Penicillium* spp. can produce MK (lovastatin) and compactin, and it has been proved that two PKSs (LNKS and LDKS, encoded by *lovB* and *lovF*), transesterase (*lovD*), enoyl reductase (ER) (*lovC*), and P450 monooxygenase (*lovA*) are involved in the biosynthesis of lovastatin (Kennedy et al. 1992; Hutchinson et al. 2000; Sorensen et al. 2003a, b). Based on the conserved DNA sequences of the KS domain of the *lovB* gene in *Aspergillus terreus*, MK biosynthetic gene cluster spanning 42 kb (GenBank accession no. DQ176595) was also cloned from a bacterial artificial chromosome library in *M. pilosus* BCRC38072 (Chen et al. 2008b). And bioinformatics analysis revealed that nine genes of MK biosynthetic gene cluster shared strong homology with the lovastatin-related and compactin-related genes in *Aspergillus terreus* and *P. citrinum*, respectively (Chen et al. 2008b), which were presumed to be required for MK biosynthesis. Disruption of *mokA* (predicted to be responsible for the synthesis of the nonaketide skeleton) caused the complete loss of MK production in *M. pilosus* BCRC38072, demonstrating the involvement of *mokA* in MK biosynthesis (Chen et al. 2008b). While deleted mutants of *mokB* (presumed to be responsible for the synthesis of the diketide skeleton) lost the ability to produce MK but accumulated intermediate monacolin J, indicating *mokB* is responsible for the diketide side chain synthesis of MK (Sakai et al. 2009). To confirm the function of *mokH* gene (predicted as an activator for MK synthesis), it was cloned and expressed in *M. pilosus* driven by the promoter of glyceraldehydes-3-phosphate dehydrogenase, and the transcript of *mokH* and MK production in the transformants were significantly higher than those of the wild-type strain. In addition, gene expression in MK biosynthetic gene cluster in the transformant appeared earlier than that in the control. These results indicated that *mokH* upregulated the transcription of MK biosynthetic genes and increased its production (Chen et al. 2010).

Pigment gene cluster

Monascus pigments (MPs), natural food colorants, usually include yellow, orange, and red, total three kinds of constituents, which have been widely used in food industries in the world, especially in Southeastern Asian countries (Feng et al. 2012; Patakova 2013). In recent years, MPs have been proved with multiple biological functions, such as anti-mutagenic and anticancer properties, antimicrobial activities, and potential anti-obesity characteristics and so on. So MPs have potential applications in different fields (Feng et al. 2012).

Table 3 Information of monacolin K and its analogs

Compound	Compound CID	SID	Chemical structure
Compactin (Mevastatin)	64715	136778553	
Lovastatin (monacolin K)	53232	160963575	
Monacolin L	3080952	135226750	
Monacolin X	125978	135081139	
Monacolin J	133310	135088664	

All these data are from the PubChem database of NCBI ▱; represents diketide side chain of MK, the left part of MK is nonaketide skeleton

MPs biosynthesis is generally considered to follow a polyketide pathway similar to citrinin and MK, but their biosynthesis pathway is unclear and controversial for a long time (Turner 1971; Jůlová et al. 1994; Feng et al. 2012). Balakrishnan et al. (2013) firstly cloned a putative MPs biosynthetic gene cluster including 24 genes based on the DNA

sequence amplified from an albino W13 with T-DNA insertions, and the expression level of *mppR1*(a transcription factor gene) and *MpPKS5* (*PKS* gene) were significantly repressed in the albino. Targeted inactivation of *MpPKS5* resulted in abolishment of pigment production, confirming *MpPKS5* is involved in pigment biosynthesis. Similarly, a Chinese research group cloned a *PKS* gene with 8.1-kb length from a transformant with T-DNA insertion (Shao et al. 2009; Xie et al. 2013), and targeted-deletion of this *PKS* gene resulted in MPs depletion. Following this, a 53-kb flanking DNA sequences of the *PKS* was cloned by SON-PCR, and a putative gene cluster related to MPs synthesis was achieved, which consisted of a *PKS* gene, fatty acid synthases gene, esterase gene, dehydrogenase gene, transport protein, and regulator gene *pigR* (data not shown). The *pigR*-deleted strain absolutely lost the capacity to produce MPs (Xie et al. 2013). Whereafter, Liu et al. (2014) demonstrated Δ*MpigE* (encoding the putative aflatoxin aldehyde reductase) mutant just yielded four kinds of yellow MPs and very little red pigments, which is different from the parent strain with productivity of red, orange, and yellow pigments, suggesting that *MpigE* may be involved in the conversion among of different pigment compositions.

Related genes to G protein signal transduction pathway in *Monascus* spp.

The production of secondary metabolites is coupled with the general development of the fungus (Yu and Keller 2005). The link between these two different physiological processes is mediated through various signaling pathways, among of which, G protein signaling pathway has been intensively studied in various filamentous fungal species, especially in *Aspergillus nidulans* (Yu 2006). The heterotrimeric G protein composed of α, β, and γ subunits play pivotal roles in the G protein signaling pathway, and each subunit is very conserved (for extensive coverage on this topic, the reader is referred to these reviews: Yu and Keller 2005; Li et al. 2007; Servin et al. 2012).

In current years, the regulation of G protein heterotrimer consisting of α, β, and γ subunits (named Mga1, Mgb1and Mgg1, respectively), and MrflbA (the regulator of Mga1) to development and secondary metabolites production was investigated by site-specific recombination in *M. ruber* (Li et al. 2010a, 2014; Yang et al. 2012), and sequence analysis revealed the deduced amino acids of these genes described above shared significant similarity with those of *Aspergillus* spp. (Li et al. 2010a, 2014; Yang et al. 2012). FadA, α subunit of the heterotrimeric G protein, enhanced vegetative growth but repressed both sexual and asexual development and sterigmatocystin production in *Aspergillus nidulans*, as well as aflatoxin production in *Aspergillus flavus* and *Aspergillus parasiticus* (Yu and keller 2005). In *M. ruber*, Mga1 as well as Mgb1 and Mgg1 have been proven to promote both sexual and asexual development and vegetative growth but repress the production of citrinin and pigment (Li et al. 2010a, 2014). Absence of MrflbA resulted in overgrowth of vegetative growth followed by autolysis and an inhibition of pigment and citrinin production and sexual reproduction in *M. ruber* (Yang et al. 2012), but increased conidial number was observed, which was different from other regulators of α subunits of G protein, such as FlbA (Lee and Adams 1994), CAG8 (Fang et al. 2007), CPRGS-1 (Segers et al. 2004), BbRGS1 (Fang et al. 2008), and AfFlbA (Mah and Yu 2006).

Monascus genome

The genome of *M. pilosus* was first sequenced and assembled by the Bioresource Collection and Research Center of Taiwan in 2004, but it hasn't been released until now. In 2010, the genome sequencing of *M. ruber* M7 was completed by de novo sequencing technique by Prof. Chen's research group, and its genomic size is approximate 23.81 Mb including 8,407 predicted genes, in which a large number of potential genes (gene clusters) were predicted but poorly characterized (He et al. 2013). Whereafter, genomic DNAs of *M. purpureus* NRRL 1596 and *M. ruber* NRRL 1597 were also sequenced using the Illumina platform and assembled with Velvet and ALLPathsLG, and the assembled size of the genome was approximate 24 Mb (http://genome.jgi.doe.gov/). The data allow us to investigate the evolution of metabolic conservatism and diversity, and even unknown genes (gene clusters) responsible for secondary metabolites and regulation, as well as development and differentiation by genome mining approach (Kaplanl et al. 2011; Lackner et al. 2012; Blin et al. 2013; Royer et al. 2013).

Conclusion and outlook

The application of molecular tool for genetic manipulation has facilitated the understanding of *Monascus* spp., but we still know a little knowledge about the physiological and biochemical processes compared with other industrial fungi such as *Penicillium chrysogenum*, *Aspergillus niger*, and *Aspergillus oryzae*, and model fungi *Aspergillus nidulans* (Sun et al. 2007; Shimizu et al. 2009; de Bekker et al. 2011; Rank et al. 2012; Weber et al. 2012). Looking for the *Monascus* nucleotide sequence submitted to NCBI data (up to Oct 25 of 2013), there are no more than 0.5 % of gene sequences involved in development and metabolism (Table 4). Therefore, it is imperative to develop the manipulation system for high-throughput gene functional analysis of the genus of *Monascus*.

Table 4 Genes cloned from *Monascus* spp. recorded in GenBank

Accession no.	Name of gene	Description	Organisms	Reference
EU118676.2	*flbA*	Regulator of G protein alpha subunit	*M. ruber*	Yang et al. (2012)
AB090877	*map*	Coding for acid proteinase	*M. purpureus*	Direct submission (2003)
FJ643483.1	No	Coding for chitin synthase	*M. ruber*	Yang et al. (2012)
KC907393.1	*laeA*	Coding for LaeA-like (*laeA*) gene	*M. pilosus*	Lee et al. (2013)
DQ178028.1	*MpLaeA*	Coding for methyltransferase	*M. pilosus*	Zhang and Miyake (2009)
AB046447.1	*aph*	Coding for acid phosphatase	*M. purpureus*	Direct submission (2002)
AB047580.1	*gap*	Coding for glyceraldehydes 3-phosphate dehydrogenase	*M. purpureus*	Direct submission (2002)
AB046446.1	*alcB*	Coding for alcohol dehydrogenase II	*M. purpureus*	Direct submission (2002)
AB046445.1	*amdS*	Coding for acetamidase	*M. purpureus*	Direct submission (2002)
AB046444.1	*niaD*	Coding for nitrate reductase	*M. purpureus*	Direct submission (2002)
KC561930.1	*pigE*	Coding for short-chain alcohol dehydrogenases	*M. ruber*	He et al. (2013)
KC561931.1	*fmdS*	Coding for formamidase	*M. ruber*	He et al. (2013)
KC561929.1	*triA*	Coding for 3-O-acetyltrans- ferase	*M. ruber*	He et al. (2013)
KC192956.1	*ku80*	Coding for the subunit of the KU protein	*M. ruber*	He et al. (2013)
KC192955.1	*Ku70*	Coding for ATP-dependent DNA helicase II 70 kDa subunit	*M. ruber*	He et al. (2013)
FJ640861.2	*macA*	Coding for adenylate cyclase	*M. ruber*	Direct submisson (2009)
FJ640864.2	No	Alternative oxidase	*M. ruber*	Direct submission (2009)
FJ640862.2	*mpka1*	Coding for cAMP-dependent protein kinase catalytic subunit	*M. ruber*	Direct submission (2009)
FJ640860.2	*mgg1*	Coding for heterotrimeric G protein gama subunit	*M. ruber*	Direct submission (2009)
FJ640855.1	No	Coding for DNA repair protein	*M. ruber*	Direct submission (2009)
DQ176595.1	No	Monacolin K biosynthetic gene cluster	*M. pilosus*	Chen et al. (2010)
FJ643482.2	No	Pyridoxine biosynthesis protein and hypothetical protein	*M. ruber*	Direct submission (2009)
DQ983312.1	*hsp90*	Coding for heat shock protein 90	*M. pilosus*	Chen et al. (2008b)
DQ206642.1	*sod*	Coding for superoxide dismutase	*M. aurantiacus*	Direct submission (2005)
DQ206643.1	*sod*	Coding for superoxide dismutase	*M. aurantiacus*	Direct submission (2005)
DQ984142.1	GAPDH	Coding for glyceraldehyde-3-phosphate dehydrogenase	*M. pilosus*	Chen et al. (2010)
Z68498.3	gpd1	Coding for glyceraldehyde-3-phosphate dehydrogenase	*M. purpureus*	Direct submission (2010)
AB167465.1	pksCT	Coding for citrinin polyketide synthase	*M. purpureus*	Shimizu et al. (2005)
KC148521.1	No	Pigment biosynthetic gene cluster	*M. pilosus*	Balakrishnan et al. (2013)
FJ640854.1	No	MFS multidrug transporter gene	*M. ruber*	Direct submission (2009)
HM191268 .1	No	Citrate synthase	*M. purpureu*	Direct submission (2010)
HM191267.1	No	Methylsterol oxidase	*M. purpureu*	Direct submission (2010)
HM191265.1	No	Pyruvate decarboxylase	*M. purpureu*	Direct submission (2010)
AB206476.1	MpMDR	Coding for a multidrug transfer	*M. pilosus*	Zhang and Miyake (2007)
AJ414729.1	Pks1	polyketide synthase	*M. purpureu*	Direct submission (2001)
FJ640859.2	mgb1	Coding for heterotrimeric G protein beta subunit	*M. ruber*	Direct submission (2009)
FJ640853.2	No	Coding for hypothetical protein	*M. ruber*	Direct submission (2009)
FJ640853.1	No	Coding for hypothetical protein	*M. ruber*	Direct submission (2009)
EU309474.1	No	citrinin biosynthesis gene cluster	*M. Aurantiacus*	Li et al. (2012)
FJ640863.1	No	Coding for cytochrome C	*M. ruber*	Li et al. (2010a)
FJ640858.1	mga1	Coding for heterotrimeric G protein alpha subunit	*M. ruber*	Li et al. (2010a)
GU723506.1	pyrG	Coding for orotidine-5'-phosphate decarboxylase	*M. Aurantiacus*	Wang et al. (2010)
JX393052.1	pigR	Pigment biosynthesis activator	*M. ruber*	Xie et al. (2013)
JX866749.1	No	Transcription factor gene	*M. purpureus*	Balakrishnan et al. (2013)
EU652942.2	No	Agmatinase gene	*M. ruber*	Direct submission (2008)
FJ643482.1	No	Pyridoxine biosynthesis protein	*M. ruber*	Direct submission (2009)
AB206475.1	Mpafr	Aflatoxin aldehyde reductase	*M. pilosus*	Direct submission (2005)

Route to improvement of gene recombination frequencies of *Monascus* spp.

It is well known that gene-targeted deletion by homologous recombination is a useful technique to study gene function in filamentous fungi. Normally, gene recombination frequencies (GRFs) of filamentous fungi are lower than 30 % (Weld et al. 2006; Meyer 2008; Kück and Hoff 2010) due to the competition with non-homologous end-joining (NHEJ). NHEJ is the dominant pathway in many eukaryotes, and its critically related genes include ku70, ku80, XRCC4, DNAPKcs, and LIG4 (Critchlow and Jackson 1998). Ku70 and Ku80 proteins by recruiting other NHEJ proteins play important roles in the NHEJ pathway, so deletion of Ku70 or (and) Ku80 can efficiently improve the GRFs of filamentous fungi (Ninomiya et al. 2004; da Silva Ferreira et al. 2006; Krappmann et al. 2006; Nayak et al. 2006; Pöggeler and Kück 2006; Meyer et al. 2007; Villalba et al. 2008; Chang et al. 2010; Li et al. 2010b). There is no exception that deletion of *ku70* and *ku80* genes can significantly improve GRFs of *M. ruber* (He et al. 2013).

Selective markers for multiple-gene functional analysis of *Monascus* spp.

During the process of gene deletion, effective selection marker is very important for rapid screening transformants. Antibiotic resistance genes and auxotrophic complementation are widely used as selection markers in genetic modification of filamentous fungi (Kopke et al. 2010). Hygromycin B (Chen et al. 2008a; Shao et al. 2009), phleomycin (Campoy et al. 2003), and aureobasidin A (Shimizu et al. 2006) have been successfully applied to the transformation of *Monascus* spp.. However, antibiotic resistance markers should be avoided for food applications from the point view of safety. So Wang et al. (2010) screened a PyrG mutant (the uridine auxotrophic strain, named UM28) from *M. aurantiacus* AS3.4384 as well as cloned the *pyrG* gene (encoding orotidine-5′-phosphate decarboxylase), and plasmid with *pyrG* gene could be transformed into PyrG mutant by using *pyrG* as a selection marker, which is available to transform *Monascus* spp. as a food-grade selection marker. In any case, the numbers of available antibiotic markers are limited, and creating auxotrophic mutants is a time-consuming process, making it difficult to delete multiple sequential genes in a single strain.

In recent years, recycled marker techniques dependent on recombinase system have been developed to replace selection markers in both prokaryotes and eukaryotes, which have broken through the limitation of selection markers in the genetic manipulation (Krappmann et al. 2005). The classical feature to this system is the ability to recombine substrate with two different or identical short DNA sequences as recognition sites. The best-exemplified members of the integrase family are the λ-integrase of bacteriophage λ (Van Duyne 2001), the recombinase Cre of *Escherichia coli* bacteriophage P1 (Abremski and Hoess 1984) and the XerCD proteins of *E. coli* (Blakely et al. 2000) and the eukaryotic FLP recombinase of *Saccharomyces cerevisiae* (Chen and Rice 2003). Up to now, FLP/FRT recombination system has been successfully applied to bacteria as well as to several eukaryotes. Kopke et al. (2010) demonstrated the recycling applicability of FLP/FRT recombination system to excise the resistance marker in *P. chrysogenum* and *Sordaria macrospora*, suggesting its applicable prospect in other filamentous fungi.

Direction to citrinin, MK, and MPs and their regulation

Although the gene clusters involving in the synthesis of citrinin, MK and MPs are isolated and analyzed by bioinformatics tools, and the effect of several genes on their production has been assessed, following questions still need to be further elucidated: (1) How many genes are directly responsible for the biosynthesis of citrinin, MKs, and MPs? (2) How does the single gene in each cluster play the role in the biosynthetic pathway? (3) What is the relationship among the metabolic pathway such as MPs, citrinin and MK? And (4) how do three kinds of MPs compounds convert? Hence, much effort must be paid to study the biosynthesis pathway described above.

It is well known that secondary metabolite production is regulated by many other mechanisms, for instance, Lae A—a global transcriptional regulator, was proved to positively regulate secondary metabolites production in several filamentous fungi (Bok and Keller 2004; Kosalkova et al. 2009). Lee et al. (2013) also attempted to overexpress an ortholog of the *laeA* gene drived by the *alcA* promoter of *Aspergillus nidulans* in *M. pilosus*, and the results revealed that MK, pigment, and the antioxidant activity of *Monascus*-fermented product from overexpressed strains were significantly increased than those of wild strain, suggesting that *Monascus* species can be improved for the production of bioactive substances by overexpressing the *laeA* gene.

In addition to the described regulation pathway, several other regulation mechanisms, such as transcription factor, histone deacetylase, DNA methyltransferase, hormone-like signaling molecules, signaling proteins such as MAP kinases and cAMP-dependent protein kinases (Fox and Howlett 2008) also participate in this process, which have laid foundation for further investigation of their functions in *M. ruber*.

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