Breeding of *Monascus purpureus* Strain KUPM5 Exhibiting Altered Production Ability of Secondary Metabolites, Citrinin and Monacolin K

（紅麹菌 *Monascus purpureus* KUPM5 株の二次代謝物質シトリニン及びモナコリン K 生産能の改変による育種）

Sittichoke Ketkaeo

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Chapter 1. Introduction

1-1. *Monascus* species

Fungi have a significant role as a source of natural product bioactive compounds (1). Since the discovery of penicillin in the 1920s, several strains have been advised to save people's lives, which is a significant sign to the exploitation of their bioactive substances (2). Filamentous fungi often produces these bioactive compounds as a result of secondary metabolites (3).

Monascus spp. are filamentous fungi belonging to the family *Monascaceae*, class *Ascomycetes*, subclass *Plectomycetidae*, order *Eurotiales*, with septate hypha and numerous branching. There could be both sexual and asexual reproduction. By sexual reproduction, ascospores are formed in a sac called Ascus. During sexual reproduction, hypha was formed to be a fruiting body, whereas ascocarps available in a range of shapes and forms, including cleistothecium, perithecium, and apothecium. Ascocarp was a homothallic that produced in two formed were antheridium and ascogonium. Nucleation fusion occurs at the proper period, followed by subsequent evolution via meiosis and mitosis (Fig. 1-1) (4). The asexual reproduction of *Monascus* spp. is diverse, it does not include nuclei or gamete fusions such as mycelium breaking, budding, sporulation resulting from nucleation division. Mitosis and conidia formation at the ends of hypha that are known specifically as conidiophores and mitospores (5).

Van Tieghem was the first to screened and defined *Monascus* spp. in red yeast rice (6). *Monascus* spp. filamentous fungi are commonly utilized in the preparation of fermented foods in Eastern Asia (7). It most well-known fermented food product is red yeast rice, which has been utilized as a dietary additive to improve the color and delicacy of meat, fish, and soybean products in Chinese cuisine. It is also renowned as a traditional medicine for health restoration and blood flow enhancement (8). Secondary metabolites produced by *Monascus* spp. have attracted interests

and have been exploited industrially in recent years due to their favorable effects on human health (7). *M. ruber*, *M. purpureus*, and *M. pilosus* are the most prevalent industrial strains (9, 10).

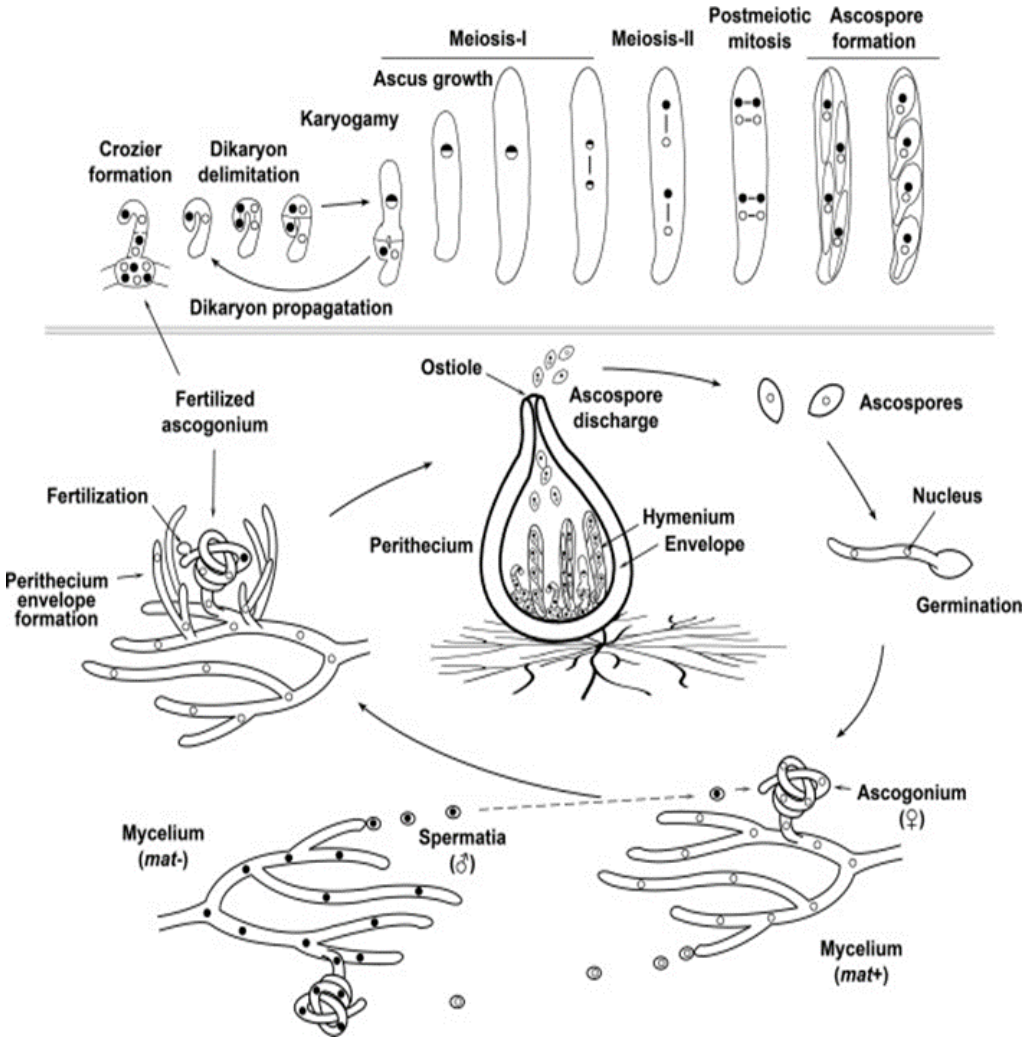

Fig. 1-1. Life cycle of filamentous *Ascomycete* fungus (4).

1-2. *Monascus* pigments

Monascus pigments are polyketide components, also known as azaphilones, that have an oxygenated bicyclic nucleus and a quaternary center (7, 11). *Monascus* species (spp), produce six pigment compounds including, rubropunctamine and monascorubramine (red pigments), rubropunctatin and monascorubrin (orange pigments), and monascin and ankaflavin (yellow pigments). The orange pigments rubropunctatin and monascorubrin are synthesized by esterifying polyketide chromophore with β-ketoacid (from the fatty acid synthase pathway) while, red pigments are produced after the orange pigments react with the amino (NH_3) group. Yellow pigments, on the other hand, are produced through the reduction of orange pigments (12) (Fig. 1-2). *Monascus* pigments have not only been employed as natural food colorants, but they also have various biological effects, including antioxidant, anticancer, antibacterial, antimutagenic, and possibly anti-obesity characteristics (7). Because the principal *Monascus* pigments are azaphilones, which inhibit other enzyme activity, they have various positive biological roles (13). Hsu *et al*. reported that *Monascus* pigments had an antiproliferative effect on HEp-2 (human laryngeal carcinoma cell line) and WiDr (human colon adenocarcinoma cell line) with no damage on normal MRC-5 and WI-38 cells (14). Tan *et al*. isolated natural yellow pigment from *M. ruber* and treated with the human breast cancer cell line MCF-7. The findings indicated that it had antioxidative properties and inhibited the migration and invasion of MCF-7 cells while causing no cell cytotoxicity. The anticancer impact might be attributed to the suppression of MMP-2 and VEGF protein expression (15).

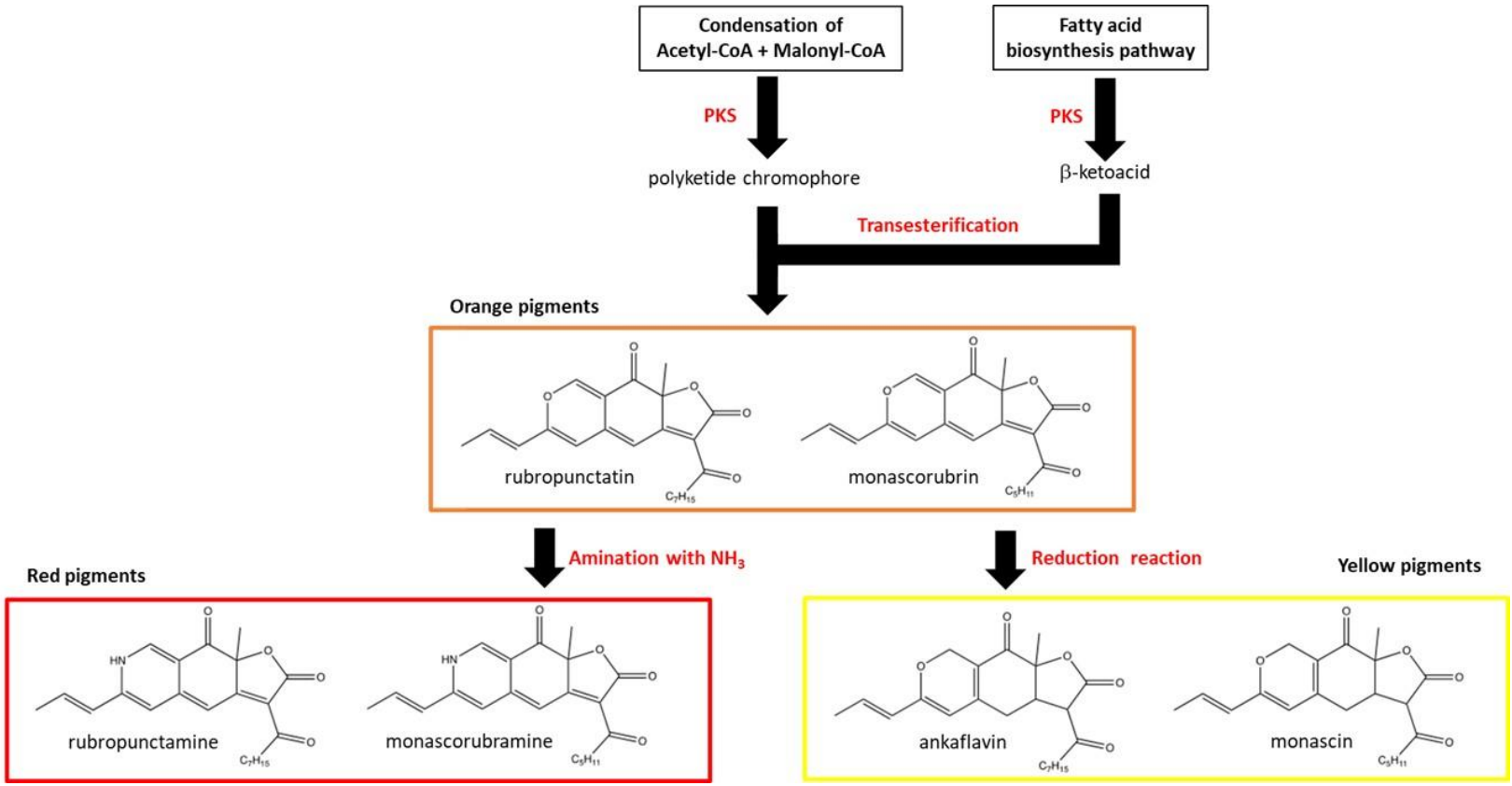

Fig. 1-2. Putative biosynthetic pathway of *Monascus* pigments and their six major pigment structures.

1-3. General information of mycotoxin produced by fungi

Mycotoxins are also secondary metabolites produced by *Aspergillus* spp., *Monascus* spp., *Penicillium* spp., and *Fusarium* spp. (16). It causes negative effects on humans and animals, and can be lethal after exposure, inhalation, skin permeation, and ingestion. Aflatoxin, citrinin, fumonisins, ochratoxin A, patulin, trichothecenes, zearalenone, T-2 toxin, deoxynivalenol, and many additional mycotoxins have been identified (16). These can be found in a variety of foods. This is the optimal environment for fungal development in many countries, particularly in locations with high humidity and high temperatures. Each toxin's effect will be distinct dependent on the type and amount of toxic substances absorbed, as well as the length of toxin exposure (17). Mokhlesi *et al*. reported that the humidity and temperature are essential parameters for fungal growth and toxin production. Some fungus for instance, *P. expansum* and *P. verrucosum* are grow at normal temperatures that are neither too hot nor too cold. (18). Type of mycotoxins is listed in Table 1-1.

Table 1-1. The fungus strains and its toxins which were produced by several species of fungi.

Fungal species	Mycotoxin	References
A. flavus	Aflatoxin	(19)
A. terreus	Aflatoxin B1	(20)
P. citrinum, *Monascus* spp.	Citrinin	(21)
A. flavus	Aflatoxin	(22)
Fusarium spp.	Zearalenone	(23)
F. moniliforme	Fumonisins	(24)
Fusarium spp.	T-2 toxin	(25)
F. graminearum,	Diacetoxyscirpenol	(26)
F. graminearum, *F. culmorum*	Deoxynivalenol	(27)

1-4. Citrinin

Citrinin, a mycotoxin, was first discovered in the 1930s by the fungus *P. citrinum* (28). There are three species of fungi could produce citrinin, namely *Monascus* sp., *Penicillium* sp. and *Aspergillus* sp. (21). Citrinin is a one of a well-known secondary metabolite derived by *Monascus* species. It has another named according to the IUPAC system is (3R,4S)-4,6-dihydro-8-hydroxy-3,4,5-trimethyl-6-oxo-3H-2-benzopyran-7-carboxylic acid (29). In 2014, the European Food Safety Authority (EFSA) informed the European commission of its scientific opinion about the health risks associated with citrinin in food and feed. The European Commission's Regulation No. 212/2014 amended Regulation No. 1881/2006 to set the maximum citrinin level in supplements derived from *Monascus purpureus* fermented rice at 2,000 µg/kg (30). However, the EFSA committee concluded in 2019 that uncertainties over the genotoxicity and carcinogenicity of citrinin continued. To protect public health, it was determined that the maximum level of citrinin in food supplements base on rice fermented with red yeast *Monascus purpureus* should be lowered to 100 µg/kg (31).

Recently, researchers have begun examining the citrinin biosynthesis pathway. Shimizu *et al*. identified a 7,838-bp polyketide synthase (PKS) gene that translated a 2593-amino acid protein with putative domains for ketosynthase, acyltransferase, acyl carrier protein, and a rare methyltransferase from *M. purpureus*. As established by gene disruption and nucleotide sequence analysis, they hypothesized that its *pksCT* gene (known as *citS*) encodes the PKS necessary for citrinin production in *M. purpureus* and act as intermediaries of the citrinin biosynthesis pathway (32). Five additional genes encoding putative dehydrogenase, $Zn(II)_2Cys_6$ transcriptional activator, oxygenase, oxidoreductase, and membrane transporter were subsequently cloned in 2007 (33).

For the production of citrinin, a minimal set of genes that involved in the biosynthesis including; (i) *citS*, encoding polyketide synthase (PKS), (ii) *citA*, encoding serine hydrolase, (iii) *citB*, encoding non heme Fe(II) dependent oxygenase (*citB*) which is involved in a oxidative ring expansion, (iv) *citD*, encoding NAD(P)$^{+}$ dependent aldehyde dehydrogenase, (v) *citE*, encoding a short-chain dehydrogenase and (vi) *citC*, encoding additional NAD(P)$^{+}$ dependent oxidoreductase (34). The step of biosynthesis is showed in Fig 1-3.

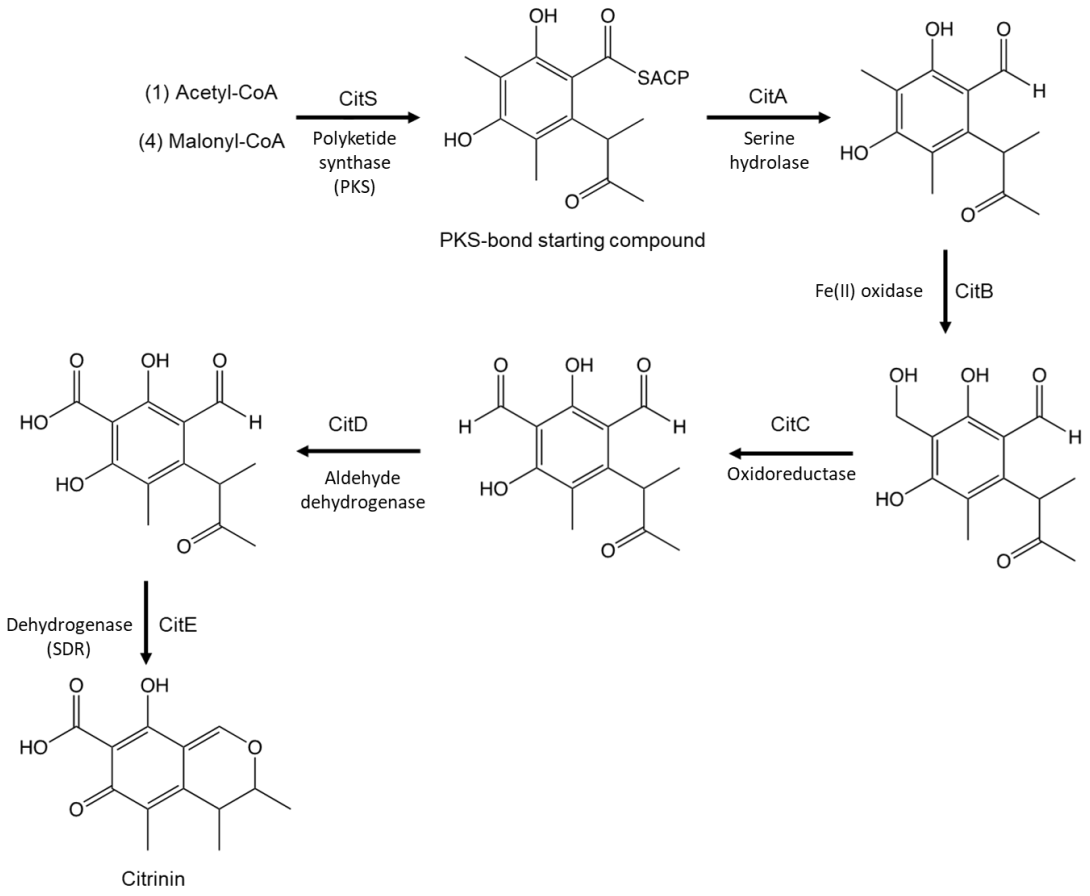

Fig. 1-3. Mechanism of citrinin synthesis from compounds in the polyketide pathway. Adapt from He and Cox, 2016 (34)

1-5. Monacolin K

Endo first isolated monacolin K from *M. ruber* culture (35). Monacolin K (lovastatin) decreases cholesterol by inhibiting HMG-CoA (5-hydroxy-3-methylglutaryl-coenzyme A) reductase, a key and rate-limiting enzyme in the synthesis of cholesterol (36). It has been reported that monacolin K was formed by the combination of their two metabolic pathways (Fig. 1-4). In one pathway, *mokA* encoded polyketide synthase (PKS) transformed acetyl-CoA and malonyl-CoA to dihydromonacolin L, which was then converted to monacolin L by oxidoreductase (*mokD*), dehydrogenase (*mokE*), and HMG-CoA reductase (*mokG*). Under the presence of P450 monooxygenase (*mokC*), monacolin L was transformed to monacolin J. In addition, acetyl-CoA and malonyl-CoA were synthesized to methylbutyryl-CoA through another pathway by polyketide synthases (*mokB*). Then, monacolin K was synthesized by the translipase (*mokF*) (37).

It has been observed that monacolin K could treat people suffering from cardiovascular diseases, namely coronary artery disease (38). Increased total cholesterol, LDL cholesterol, and triglyceride levels, as well as a decrease in HDL cholesterol, are all important independent predictors of coronary artery disease, and statins might aid with this (39). Statins, a commonly prescribed family of cholesterol-lowering medications, function by competitively blocking the rate-limiting enzyme, HMG-CoA reductase, to treat hyperlipidemia (40). With the predominance of hyperlipidemia, *Monascus* fermented food product have been identified as a hypolipidemic function food with cholesterol-lowering properties. Lin *et al*. found that fermented rice produced by *M. purpureus* Went dramatically lowered LDL-cholesterol, total cholesterol, triglycerides, and apolipoprotein B levels while also being well tolerated in hyperlipidemic individuals. As a result, the *Monascus* fermented food product has been recognized as a functional food for lowering cholesterol (39).

The most frequent adverse effects of lovastatin include, in roughly decreasing order of frequency: creatine phosphokinase increase, flatulence, abdominal pain, constipation, diarrhoea, muscular aches or pains, nausea, indigestion, weakness, blurred vision, rash, dizziness, and muscle cramps. As with all statin medicines, it can induce myopathy, hepatotoxicity (liver damage), dermatomyositis, or rhabdomyolysis in rare cases. This can be fatal if not recognized and treated soon, thus any inexplicable muscular discomfort or weakness while taking lovastatin should be reported to the prescribing doctor right once. Other unusual adverse effects that should be reported immediately to either the prescribing doctor or an emergency medical agency include (41).

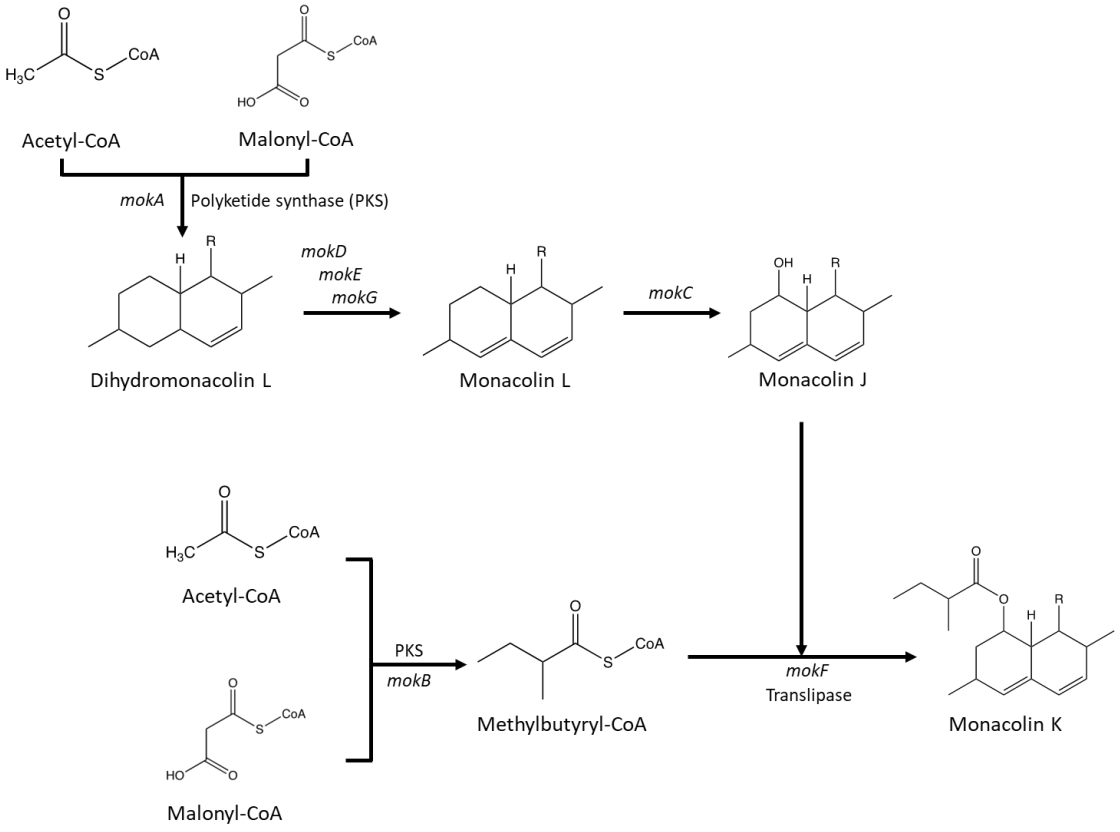

Fig. 1-4. Monacolin K biosynthetic pathway in *Monascus* spp.

1-6. Synchrotron light irradiation

Synchrotron light is an electromagnetic wave, precisely like sunlight. Unless, they are emitted by charged particles such as electrons, which travel at near-light speed and are subjected to electromagnetic radiation. When an arc is pushed to be fed by a magnetic field, the electrons lose energy and release it as electromagnetic waves. It also includes a wide variety of continuous wavelengths such as infrared, visible light, ultraviolet, and X-rays. This enables scientists to determine the most appropriate wavelength or energy for usage in diverse study disciplines as required (42).

Synchrotron light production consists of four major processes including; (i) Generating electrons by passing an electric current through the metal filament until it warms up and emits electrons. The high voltage anode is then used to draw the electrons in the same direction. (ii) Acceleration of electrons in a straight line using a linear particle accelerator (or linac) to achieve a high speed (40 MeV). This electron is then fed into an accelerator. (iii) Electrons are accelerated in a circular motion to achieve speeds close to the speed of light (1 GeV). (iv) The final stage, electrons are transferred to the electron storage ring. The electron storage ring is used to boost electron accelerators to 1,200 million electron volts (1.2 GeV) in order to produce synchrotron light by forcing electrons to diffraction and release light, or photons emitted during diffraction, and electrons are produced to deliver the light to various fields of study (42-45) (Fig. 1-5).

Synchrotron is now being used in genetics research and is becoming a modern alternative to research potential development, especially in line improvement and development a certain species of plants and organisms used in the food industry. Sakamoto and coworkers used synchrotron ion beams at concentrations of 1, 3, 5, and 10 Gy to alter *Lactuca sativa*, a vegetable (lettuce) to develop a viable strain that may enhance lettuce production rate for a limited time. It was discovered that using synchrotron light at concentrations less than 5 Gy was capable of producing new lettuce cultivars that could grow quickly (46). Furthermore, they demonstrated that

synchrotron light is an effective to breeding organisms by experimenting with synchrotron-induced light to produce phenotypic changes. *Chrysanthemum* is a famous ornamental plant that is in high demand in the market. According to the results, new strains of *Chrysanthemum* with attractive and unique colors were developed using synchrotron light at concentrations ranging from 11 to 23 Gy (47).

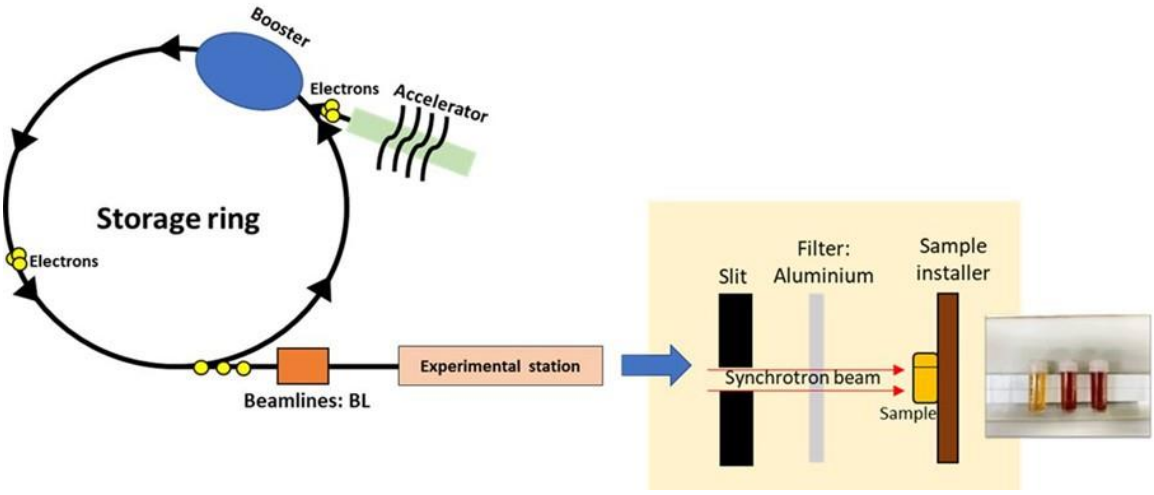

Fig. 1-5. The scheme of a synchrotron light source facility. The electron is accelerated in the accelerator to produce a high voltage light source. After that, a relevant photon is emitted and chosen by the beamline station for each experiment. The sample is fixed in place by a holding bar, and a synchrotron beam is projected in a horizontal plane using an aluminum sheet as a filter.

1-7. Genome editing by CRISPR/Cas9 system

All living things have natural enemies in addition to microorganisms. The natural enemies of bacteria are bacteriophages, or phages, a kind of virus with a vast range of species and strains found in a variety of habitats. When phages infect bacterial cells, they proliferate by utilizing the bacterial cell's own processes to develop components that then destroy the bacterial cells. However, bacterial have their own defensive systems against phages and genetic material, such as changing receptor molecules on the cell surface to inhibit phage attachment and transferring genetic material into another cell. One well-known example is restriction endonuclease enzymes, which degrade invading foreign DNA, but bacteria protect their DNA from particular cutting enzymes by altering their own DNA molecules by adding strands of complementary DNA. The methyl group is positioned at the precise enzyme termination location. This procedure is known as methylation. This recently discovered process resembles the innate immune system seen in common vertebrates (48). Interestingly, another novel bacterial immunity known as Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) has been discovered.

The clustered regularly interspaced short palindromic repeats (CRISPR) associated protein 9 (Cas9) system known as CRISPR/Cas9, a bacterial defense mechanism against phage infection and plasmid transfer in nature has become a powerful genome editing tool due to its high efficiency and ease of usage (49). The CRISPR immunity mechanism consists of three distinct stages (50) (Fig. 1-6). In the first stage, known as "adaptation" or "immunization," a piece of phages or plasmid DNA is introduced into the CRISPR region. Cas 1 and 2 proteins facilitate this process. The novel sequence is recognized by Cas proteins. The phage-specific nucleotide, known as the Protospacer Associated Motif (PAM), is positioned in close proximity to the protospacer region that would be cut and reintroduced into CRISPR. In the second stage, known as "CRISPR expression," all CRISPR nucleotide sequences are transcribed into RNA, also known as pre-crRNA. In the third

stage, endoribonucleases Cas proteins cut the pre-crRNA into small crRNAs. The crRNA is a phage DNA-specific spacer region. It would adhere to the Cas protein and carry them to the specific phage DNA, where bind and destroy phage DNA (51).

The CRISPR/Cas9 system includes two key components: Cas9 endonuclease and single-guide RNA (sgRNA) (52). Cas9 is derived from the bacteria *Streptococcus pyogenes*, and the sgRNA has a 20-nucleotide sequence. To properly construct the CRISPR/Cas9 system in the target location of the genome, the nuclease must include both Cas9 endonuclease and sgRNA (53). Cas9 then detects a protospacer adjacent motif (PAM) sequence and catalyzes double-strand breaks (DSB) at the target site (54). CRISPR Cas9 genome editing allows to change the gene of interest by deleting, inserting, or replacing DNA in a sequence-specific manner (55). The initial method of genome editing involves inserting DSBs at the target region of a certain gene. Because DSBs are the most common type of homologous recombination, they have been used to purposefully and accurately modify the genome in fungal molecular biology. When DSBs occur at the target location of a gene, the damaged DNA is repaired using either the non-homologous end joining (NHEJ) or the homologous direct recombination (HDR) pathways (53). The primary distinction between the two repair processes is that non-homologous end joining (NHEJ) binds DNA without homology, whereas homology-directed repair (HDR) needs homologous or homologous sequence stretches. Cas9-induced DSBs in mammalian cells are repaired mostly by NHEJ and partially through HDR, which involves gene conversion, microhomology-mediated end joining (MMEJ), and single strand annealing (SSA) (56). NHEJ, being an error-prone repair process, produces a high frequency of insertions or deletions (indels) at the repair junctions. The HDR process is triggered in the presence of a donor template to induce particular insertions, deletions, or mutations (57). A homologous DNA fragment (donor DNA) to the target sequence is required during the HDR process (53).

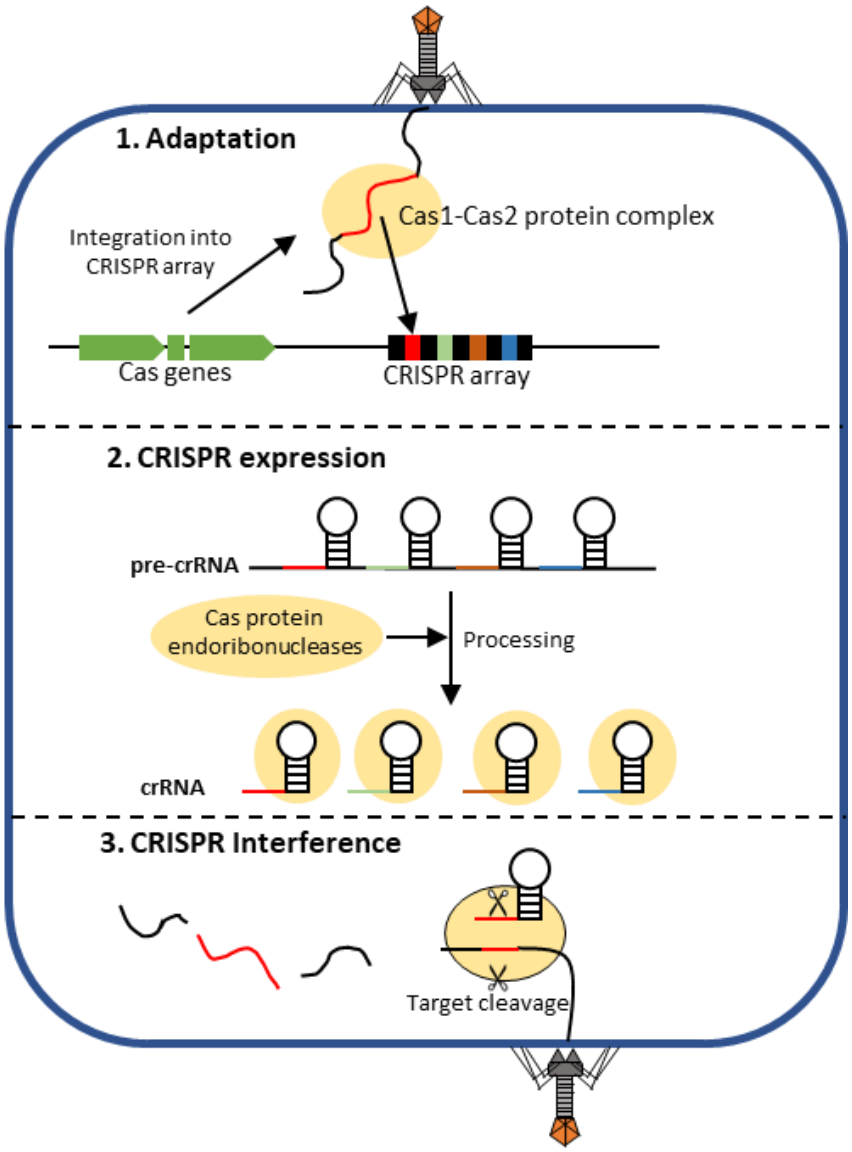

Fig. 1-6. The three stages of CRISPR immunity systems in bacteria cell.

1-8. General information of strain improvement of filamentous fungi

In addition to typical food fermentation industries, filamentous fungi are widely used in industrial processes for the manufacture of enzymes, antibiotics, organic acids, and other bioactive substances (58, 59). Strain improvement is a fundamental aspect of process development that generally aims to decrease production costs or enhance an output potential. Higher enzyme and biomass yields, superior physiological features, and effective utilization of a variety of industrially relevant substrates are all objectives of genetic strain improvement. The practical possibilities for strain improvement are becoming more diverse, depending on the nature of the product's use, human consumption patterns, or regulatory restriction (60). Furthermore, the availability of biological molecular tools, as well as understanding of filamentous fungus genetics and physiology, would affect fungal species improvement strategies (61). Biological methodologies for the development of fungal strains generally include a variety of techniques. One of traditional method is using chemical and physical mutagens to induce random mutations in the genome. Alternatively, genetic engineering can be used to activate gene modification by inhibiting or enhancing the gene activities. It is prevalent because to its time-saving and precise nature, but there are still legal concerns over its use in the food industry (62).

1-8-1. UV-mutation, NTG treatment, and synchrotron light irradiation

The main determinant of all genetic variation is mutation. The efficacy of induced mutation is determined by the kind of base damages including insertion, deletion and etc., which caused by a particular mutagen to DNA and the cellular mechanism involved in its repair systems. Chemical mutagenesis–DNA alkylating by NTG (N'-methyl-N'-nitro-N'-nitrosoguanidine), which often causes a phenylephrine-based mutation, is one of the most commonly employed mutations for fungal strain improvement (63). Wan *et al*. have been applied the chemical mutagenesis by NTG to improve the *A. oryzae* ATCC 22788 strain that can be enhance kojic acid production, an essential substance for cosmetic industry. They successfully obtained mutant strain which was 100-fold higher production than that in the wild type (64). Heerd *et al.* used cycle mutagenesis to induced a mutation in *A. sojae* ATCC 20235 to develop polygalacturonase hyper producing strain by continuously UV irradiation at 254 nm, 5 min for total 3 cycles of exposures. The results showed mutant strain DH56 enhanced 2.4-fold in polygalacturonase production at the highest activity about 98.8±8.7 U/ml (65).

In addition to effectiveness, UV irradiation induces the formation of pyrimidine dimers, which leads to mutation and point deletion, which the more safety due to the lack of chemicals contaminated. User safety is a key feature of the usage of chemical and physical mutagens. This is not the case with long-lived and very strong chemical mutagens like NTG, which need the use of protective equipment and neutralization for disposal. Physical mutagens, such as UV that provide a lesser danger to the user, might be chosen. Kalaivani and Rajasekaran successfully obtained *M. purpureus* non-citrinin production mutant strain 254 by using UV irradiation at 254 nm lesser than 25 min of exposure time, and increased in monacolin K production of about 74.32% (66). Nahideh *et al.* aimed to enhance the cellulase production yield for an application in enzyme industry. They used *A. niger* PTCC 5162 as wt. After treated with UV irradiation for 220 sec, the

mutant was cultured on rice and wheat straw for 10 days. The resultant mutant produced two-times higher production in cellulase activity.

Recently, synchrotron light has become a novel mutagenic agent for strain breeding. The most remarkable feature of synchrotron radiation is its tremendous intensity, which is several orders of magnitude more than that of normal X-rays. Thus, synchrotron light has several advantages, notably in the fields of materials science, complex materials physics, and medicine (67). Tanaka and coworkers investigated the mutation rates on higher plant *Arabidopsis*, *Chrysanthemum* and *Carnation* phenotypes by carbon ions which were 20-fold higher than by electrons. These results showed that the features of ion beams for mutation induction include a high mutation frequency and a wide mutation spectrum, resulting in the effective induction of new mutants. In contrast, PCR and sequencing tests revealed that 50% of all mutants derived by ion beams had large DNA changes, whereas the other 50% showed point mutations. It is likely that ion beams induced a small amount of extensive and permanent DNA damage, leading to the generation of a null mutation with a novel mutant phenotype (68).

1-8-2. Genome editing of filamentous fungi

Filamentous fungus is necessary to sustain life, industrial and agricultural production. With the increased availability of whole genomes for fungal species, the study of filamentous fungi has opened up new avenues for gene modification. Currently, the filamentous fungi employed to produce enzymes and recombinases are mostly *Aspergillus* species. Filamentous fungi produce a large amount of secondary metabolites, which have grown more significant. Zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the CRISPR/Cas systems are all used in modern genome editing methods. Despite the fact that ZFN technology predates TALENs and CRISPR/Cas, it is rarely used on filamentous fungi because to the labor-intensive approach. In *A. oryzae*, Li *et al*. (69) successfully established a CRISPR/Cas9 system for gene knockout, targeting the kojic acid production genes *kojA*, *kojR*, and *kojT*. After transformation, the mutational rates were 50%-75%, and kojic acid production was reduced by more than 90% compared to the parental strain.

Fungal azaphilones have attracted the interest of researchers in the case of *Monascus* species owing to their significant potential in the food and pharmaceutical industries. However, there is a significant bottleneck owing to low production in particular strains and the ability to genetically engineer azaphilone-producing fungus. Duan and coworkers reported a systematic metabolic engineering technique for increasing *Monascus* azaphilone synthesis in *M. purpureus* HJ11. The researchers methodically constructed a combination of promoter engineering, gene deletion, rate-limiting enzyme overexpression, inhibition of the competing route, enzyme engineering, and metabolic rebalancing. The highest yield of *Monascus* azaphilones was effectively enhanced to 906 mg/g and 14.6 g/L, 2.6, and 3.7 times higher than those wt, respectively (70).

1-9. Purpose of this study

Food perspective has changed dramatically in recent years. It should provide additional health advantages in addition to their nutritional value. Functional foods may promote development and reduce the risk of chronic illness. Thus, worldwide functional food markets are predicted to grow as a result of increased demand for functional foods caused by unhealthy and food consumption (71). *Monascus* fermented food products, particularly red koji (also known as red yeast rice), have received more attention than ever before due to their verified efficacies in lowering cholesterol levels and having other positive benefits on human health. However, safety over *Monascus* fermented food product is still concerning and controversial due to citrinin, which limits *Monascus* fermented food products to be consumed. In consequence, it is important to develop citrinin-low/or free strain to be used as industrial strain. In this research, I used *M. purpureus* strain KUPM5 as the starting strain throughout the experiments. Strain KUPM5 was isolated from Thai fermented food, *sufu*, in a Thai local market. This strain has hyperpigmentation properties especially in red pigments (72).

In Chapter 2, traditional mutagenesis methods, such as UV irradiation and NTG chemical treatment, were applied to induce the mutations in *M. purpureus* KUPM5 that resulted in mutant strains with decreased citrinin synthesis while retaining enzyme function for *sufu* production. In order to evaluate mutant strains appropriate for red koji production in the food industry.

In Chapter 3, our concerned goal is the genomic information of *Monascus* species is limited and unclear when compared to another filamentous fungi. Thus, we carried out *de novo* analysis with a suitable assembler, utilizing an assembly method that included short reads and long reads. We completed the clarification of *M. purpureus* KUPM5 genomic information. Following that, gene annotation and prediction were investigated as well as the gene clusters of secondary metabolites and their functions.

In Chapter 4, we would like to improve an ideal strain showing higher production of Monacolin K. Thus, synchrotron light irradiation, an alternative mutagen was applied to induce mutations in *M. purpureus* KUPM5 in order to obtain MK-hyperproducing strains. We next performed comparative genomic analysis on selected mutants against parental strains to discover the mutation pattern, derived by an alternative potent physical mutagen, synchrotron light. We proved that synchrotron light induced mutation showing a high efficiency for strain breeding in *M. purpureus.*

In Chapter 5, CRISPR/Cas9 genome editing would be used to develop the superior *Monascus* strain from Chapter 4. First, the transformation system for *M. purpureus* KUPM5 were developed. By targeting the *citS* gene, which is responsible for citrinin synthesis, the CRSPR-Cas9 system was introduced into the mutant strains with high MK production. This enables the mutants to suppress the *citS* function, leading in a decrease in citrinin production. Finally, using genome editing to construct a *Monascus* strain with high MK production allows for the creation of superior *Monascus* strains with a high level of functionality and safety.

Chapter 2. Induced mutation of *Monascus purpureus* KUPM5 to develop a low citrinin producing strain for use in the red koji industry

2-1. Introduction

Red koji (red mold rice) has been used as food additive and colorant in the food industry. It is produced from *Monascus purpureus* and has been traditionally used to produce several fermented products, such as red soy cake, red sufu, sour pork, fish sauce, and rice fermented wine in east Asia (73). It has several beneficial effects such as cancer prevention and blood sugar level reduction as well as anti-inflammatory and antitumor effects (74). However, certain *Monascus* sp. produce various amounts of citrinin—secondary metabolite mycotoxin. Citrinin has negative effects in humans and animals, including nephrotoxic, hepatotoxic, and cytotoxic effects (75-77). It also exhibits antibacterial activity (78).

In recent years, there has been a greater awareness of the consequences of citrinin to human health. The European Food Safety Authority has established the maximum level of citrinin in food supplements, based on the red koji, as 2000 μg/kg because of concerns that citrinin directly affects kidney toxicity (79). Therefore, the selection of *Monascus* strains is the first important step in the production of red koji with low amounts of citrinin. Citrinin biosynthesis occurs in *Monascus*, *Penicillium,* and *Aspergillus* species (21). The pathway involves the synthesis of an unreduced trimethylated pentaketide by a non-reducing polyketide synthase (nrPKS), known as CitS. *cit* cluster centered on the 7.9 kbp gene, which encodes an iterative type I non-reducing polyketide synthase (CitS), was discovered in *M. purpureus.* (33, 80). The disruption of *citS* resulted in the complete elimination of citrinin production in *M. purpresus* (81). Thus, the construction of citrinin-nonproducing *Monascus* strains using gene recombinant technology is a powerful tool, although using classical mutagenesis might be more favorable for use in the fermented food industry.

In the present study, we induced mutations in *M. purpureus* KUPM5 isolated from Thai fermented food, sufu (72), as a parental wt strain by ultraviolet (UV) irradiation, N-methyl-N′-nitro-N-nitrosoguanidine (NTG) treatments, and a combination of UV and NTG to produce mutant strains with low citrinin production. The selected mutant strains were characterized with respect to the citrinin production, red pigments, and enzyme activity to evaluate mutant strains suitable for production red koji in the food industry.

2-2. Materials and methods

2-2-1. Microorganisms and culture media

M. purpureus strain KUPM5 isolated from Thai fermented food, *sufu* (72), was obtained from the Faculty of Agro-industry, Kasetsart University, Thailand. *B. subtilis* NBRC13719 was obtained from the National Bioresource Center, Japan. KUPM5 was maintained on potato dextrose agar (PDA) medium (Becton Dickinson company) at 30°C for 7 days. *B. subtilis* strain was maintained in Luria–Bertani (LB) broth.

2-2-2. Red koji production

Red koji was prepared using the procedure described by Kanlayakrit *et al*. (72). Fifty grams of long grain rice (*Oryza sativa*) were soaked in tap water for 8 h. Excess water was drained, and soaked rice was transferred into a 500 ml Erlenmeyer flask, after which 15 ml of distilled water was added and then autoclaved at 121°C for 30 min. After the steamed rice was cooled at room temperature, 2 ml of fungal spore suspension (10^6 spores/ml) was inoculated and incubated at 30°C for 7 d. The resultant red koji was stored at −80°C until use.

2-2-3. Mutagenesis

UV irradiation: *Monascus* spore suspension was prepared from *Monascus* grown on PDA plates with 0.01% Tween80 solution, and the number of the spores was adjusted to 10^6 spores/ml. Four milliliter of spore suspension was poured in a dish (d.i. 90 mm), which was then placed under UV lamp at a distance of 55 cm for 20, 30, and 40 min (82). The spores were collected by centrifugation and suspended in fresh potato dextrose broth (PDB) medium. The spores were incubated at 30°C in the dark for 6 h and then spread on PDA plates at 30°C for 72 h. Colonies developed, which were then screened by plate bioassay.

NTG (N-methyl-N′-nitro-N-nitrosoguanidine) treatment: The number of spores was adjusted to 10^6 spores/ml using 0.1M citrate buffer (pH 5.0). Two milliliters of an NTG solution (50, 100, and 150 μg/ml) was added to 2 ml of spore suspension. After incubation for 30 min, the spores were immediately collected by centrifugation and washed twice with sterilized distilled water. The spores were grown in fresh PDB medium at 30°C for 6 h and spread on PDA plates and then incubated at 30°C for 72 h. Colonies developed, which were then screened by plate bioassay.

Combination of UV irradiation and NTG treatment: Two milliliter of an NTG (100 μg/ml) solution was added to 2 ml of spore suspension and placed under UV lamp for 10, 20, and 30 min. The spore suspension was centrifuged and washed twice with sterilized distilled water. The spores were incubated in PDB medium at 30°C for 6 h and spread on PDA plate and then incubated at 30°C for 72 h. Colonies developed, which were then screened by plate bioassay.

2-2-4. Screening of *Monascus* mutants showing low citrinin production

Screening of *Monascus* mutants by a plate bioassay was performed according to the method described by Wang *et al.* (82). Mutagenized *Monascus* cells were spread on PDA plates containing 0.01% TritonX-100 and incubated at 30°C for 72 h. *B. subtilis* strain NBRC 13719 was grown in LB medium at 30°C for 24 h. One hundred microliter of *Bacillus* culture was added to 8 ml of soft LB agar medium (0.7% agar) and then poured onto the *Monascus*-grown plate. After 72 h, mutant strains with low citrinin production were selected by observing bacterial inhibition zones around fungal colonies. The inhibition ratio was calculated using the following formula:

$$Inhibition\ ratio\ (\%) = \frac{C - E}{C} \text{ x } 100$$

where *C* is the average zone of inhibition of the wt and *E* is the average zone of inhibition of the mutant.

2-2-5. Determination of amounts of citrinin in the koji using HPLC

Red koji was prepared using the selected mutant strains. Citrinin produced by the *Monascus* strains in the koji were extracted by the method described by (83). One gram of dried red koji was ground and extracted with 40 ml of 70% ethanol by shaking at 80 rpm at 30˚C. After 3 h, the extracts were filtrated through a membrane (Dismic-13CP, 0.45 μm pore size, Advantec Inc., Japan), and the amounts of citrinin in the red koji was determined by HPLC. HPLC was performed using a C18 column (TSKgel ODS 4.6 mm x 250 mm, Tosho, Japan) and 80% acetonitrile:0.1% phosphoric acid (60:40) as the mobile phase at a flow rate of 1.0 ml/min, with a fluorescence detector (excitation at 330 nm and emission at 500 nm).

2-2-6. Determination of the amount of red pigments

Red pigments in the koji were extracted according to the method described above in citrinin extraction. An amount of red pigments in the koji was estimated by measuring absorbance at 500 nm. Results were expressed as absorbance unit per gram koji (83).

2-2-7. Enzyme assays

Water (150 ml) was added to the red koji (7 d growth), and enzymes were extracted at 4°C for 4 h. The resultant crude enzyme solution was filtrated through a membrane (Whatman No.1, 125 mm) and stored at −20°C (72).

α-Amylase assay: α-Amylase activity was determined using the method described by Onishi and Sonoda (84). A substrate solution (3.9 ml of 1% gelatinized starch in 0.1M Mcllvaine buffer, pH 4.0) was preincubated at 50°C for 10 min and then added to 0.1 ml of the crude enzyme solution. After 10 min, 0.2 ml of the reaction mixture was added to 5 ml of 0.167 mM iodine solution. The α-amylase activity of the red koji was determined at absorbance about 770 nm. One unit (U) of α-amylase was defined as the change in absorbance per 10 min.

Protease assay: Protease activity was estimated according to Chancharoonpong *et al*. (85) using casein as a substrate. A substrate solution (1 ml of 1.5% casein in 0.1M phosphate buffer pH7.0) was preincubated at 37°C for 10 min. One milliliter of the crude enzyme solution was added, well shaken, and incubated at 37°C for 10 min. A 2 ml of 0.44M trichloroacetic acid (TCA) solution was added to terminate the reaction and then incubated for 30 min. Then 2 ml of the mixture was filtrated and then 2.5 ml of 0.4M Na_2CO_3 solution was added and well shaken, and 0.5 ml of Folin reagent was added. Protease activity was determined by measuring the absorbance at 660 nm. One unit (U) of protease activity was defined as the amount of 1 μg tyrosine released per minute.

Lipase assay: Lipase activity was assayed using Stuckmann's method (86) with *p*-nitrophenyl palmitate (*p*-NPP) as a substrate. The substrate solution was prepared by mixing solution A (10 ml of 30 mg *p*-NPP in isopropanol) with solution B (90 ml of 0.05M phosphate buffer, pH 7.0, containing 100 mg arabic gum (acacia), 207 mg sodium deoxycholate, and 2 ml TritonX-100). The substrate solution (2.4 ml) was preincubated at 37°C for 10 min, and 0.1 ml of the crude enzyme solution was then added and incubated at 37°C for 15 min. Lipase activity in the red koji was determined by measuring absorbance at 410 nm. One unit (U) of lipase activity was defined as 1 μmol of *p*-nitrophenol released per minute.

2-3. Results

2-3-1. Selection of the low citrinin producing mutants

M. purpureus KUPM5 (wt strain) was isolated from Thai fermented food, *sufu* (72). To make this strain safe for producing red koji, we attempted to improve this strain by reducing citrinin production. UV irradiation, NTG treatment, and the combination of both UV irradiation and NTG treatment were used to induce the mutation in wt KUPM5. The irradiation period of UV and concentration of NTG treatment were determined by the survival rate of the wt as an index. After UV irradiation for 30 and 40 min, the survival rate of wt strain was 10%, and 7%, respectively. Meanwhile, the spores treated with 50, 100, and 150 μg/ml NTG showed 50%, 10%, and 0% survival, respectively. The survival rate of spores after UV irradiation for 10 min in the presence of 100 μg/ml of NTG was <10%, whereas spores under UV treatment for 20 and 30 min showed 0% survival. We determined the mutagenic conditions to wt strain KUPM5 with UV irradiation to be 30 min and with 100 μg/ml NTG treatment to be 30 min.

In total, 2,420 colonies, including 983 colonies by UV irradiation, 850 colonies by NTG treatment, and 587 colonies by the combination of both UV and NTG, were screened by plate bioassay using *B. subtilis* NBRC13719. Ten colonies (UV: 2, NTG: 4, and combination of UV and NTG: 4) were selected as showing >90% fungal colony, and the size of bacterial inhibition zone was smaller than that of the wt (Fig. 2-1). These 10 mutants were serially subcultured 5 times on the PDA medium to stabilize the mutation.

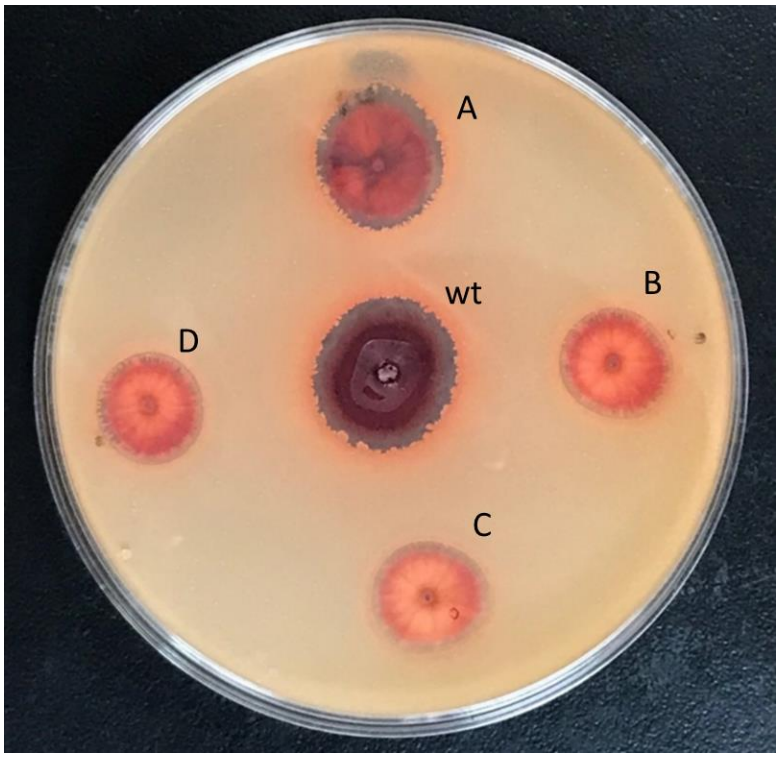

Fig. 2-1. Zone of inhibition of *Monascus* mutant strains against *Bacillus subtilis.* All mutant strains were cultured at 30°C for 5 d on PDA plate, and *Bacillus* culture (in soft LB agar medium, 0.7% agar) was poured onto the plate for screening. Mutant strains KS301U (A), KS1001C (B), KS1003C (C), and K1004C (D) showed lower citrinin production because of the smaller zone of inhibition compared with the wt strain KUPM5 (center).

Table 2-1. Size of inhibition zone and inhibition ratio of *Monascus* mutants.

***M. purpureus* strains**	**Size of inhibition zone (cm)**	**Inhibition ratio (%)**
KUPM5 (wt)	0.24	-
KS301U	0.15	37.5
KS302U	0.16	33.3
KS101N	0.12	50.0
KS102N	0.07	70.8
KS103N	0	100
KS104N	0.05	79.2
KS1001C	0.07	70.8
KS1002C	0.02	91.7
KS1003C	0.05	79.2
KS1004C	0	100

2-3-2. Citrinin production in the koji produced by mutants

Red koji was individually produced by the selected 10 *Monascus* mutant strains. Citrinin was extracted from the red koji and analyzed by HPLC to confirm citrinin production levels in the mutants (Fig. 2-2). Among these 10 mutants, all mutants KS1001C, KS1002C, KS1003C, and KS1004C obtained after the combination of UV and NTG treatments completely eliminated citrinin production in the red koji (Table 2-2). Strains KS301U and KS302U showed citrinin productivity of 32.0±0.3 and 33.2±0.2 ng/g koji, respectively, which was 80% lower than that of the wt (174.3±1.5 ng/g koji). However, strains KS101N, KS102N, KS103N, and KS104N, selected after NTG treatment, produced higher amounts of citrinin in red koji than the wt (Table 2-2).

Table 2-2. Citrinin concentration and Relative citrinin production of *Monascus* mutants.

***M. purpureus* strains**	**Citrinin concentration (ng/g)**	**Relative citrinin production to wild type**
KUPM5 (wt)	174.3±1.5	100%
KS301U	32.0±0.3	18.4%
KS302U	33.2±0.2	19.1%
KS101N	5347.3±17.1	3067.7%
KS102N	3745.4±19.4	2148.7%
KS103N	3452.4±89.7	1980.6%
KS104N	2229.0±32.2	1278.8%
KS1001C	0	0
KS1002C	0	0
KS1003C	0	0
KS1004C	0	0

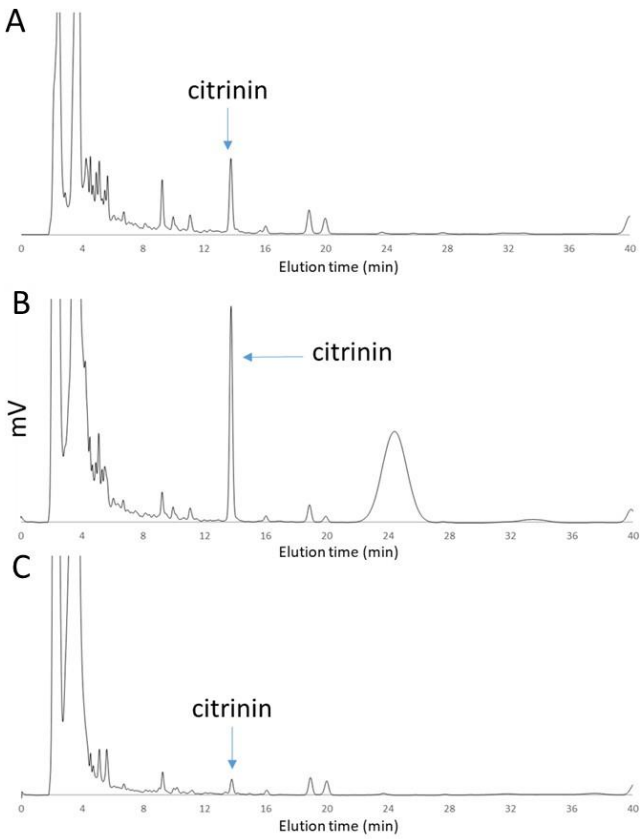

Fig. 2-2. HPLC chromatogram of citrinin in koji extracts from strain KUPM5 (wt) (A), KS104N (B), and KS301U (C). The retention time of citrinin was detected at 13.7 min.

2-3-3. Pigment production

M. purpureus produces the red pigment, azaphilone, as a secondary metabolite (12). This properties of *Monascus* allows it to be used as a food colorant. The result of red pigment production is shown in Table 2.3. Strains KS103N and KS104N produced red pigments at levels of 10.03±0.18 and 14.27±0.86 U/g koji, respectively, higher than the wt (9.17±0.41 U/g koji). Strain KS302U maintained red pigment production with 9.05±0.80 U/g koji, which was similar to the wt. KS301U produced 24% less red pigment than the wt at 6.95±0.30 U/g koji, whereas all mutants KS1001C, KS1002C, KS1003C, and KS1004C obtained by the combination of UV and NTG showed more than 70% reduction in red pigment production. Thus, combination mutants almost lost the ability to produce red pigments and became albino mutants (Fig. 2-3).

2-3-4. Enzyme activities in red koji produced by *Monascus* mutant strains

The quality of Thai fermented food *sufu* (red soybean curd) is closely related with activities of α-amylase, protease, and lipase from red koji (87). Therefore, it is important to obtain mutants showing these enzymatic activities. To characterize the mutant strains, enzyme activities in the red koji extract of selected mutants were determined (Table 2-3). The red koji extract produced by strains KS301U and KS302U showed 4.51±0.65 U/ml and 4.26±0.22 U/ml of α-amylase, respectively, which were similar to α-amylase activity of the wt (4.89±0.65 U/ml). As compared to enzyme activities of wt (2.63±0.67 U/ml of protease, 5.39±0.27 U/ml of lipase), strains KS301U and KS302U exhibited enhanced activities with 3.38±0.30 U/ml of protease, 9.21±0.73 U/ml of lipase, and 6.43±0.72 U/ml of protease, 12.72±0.27 U/ml of lipase, respectively. The red koji made by the mutant strain KS104N showed 4.39±0.22 U/ml of α-amylase and gave the high activities of lipase (13.60±1.00 U/ml) and the highest activities of protease (20.09±1.06 U/ml). The mutant

strains KS102N, KS1002C, and KS1003C produced lower α-amylase activities and higher lipase activity than the wt.

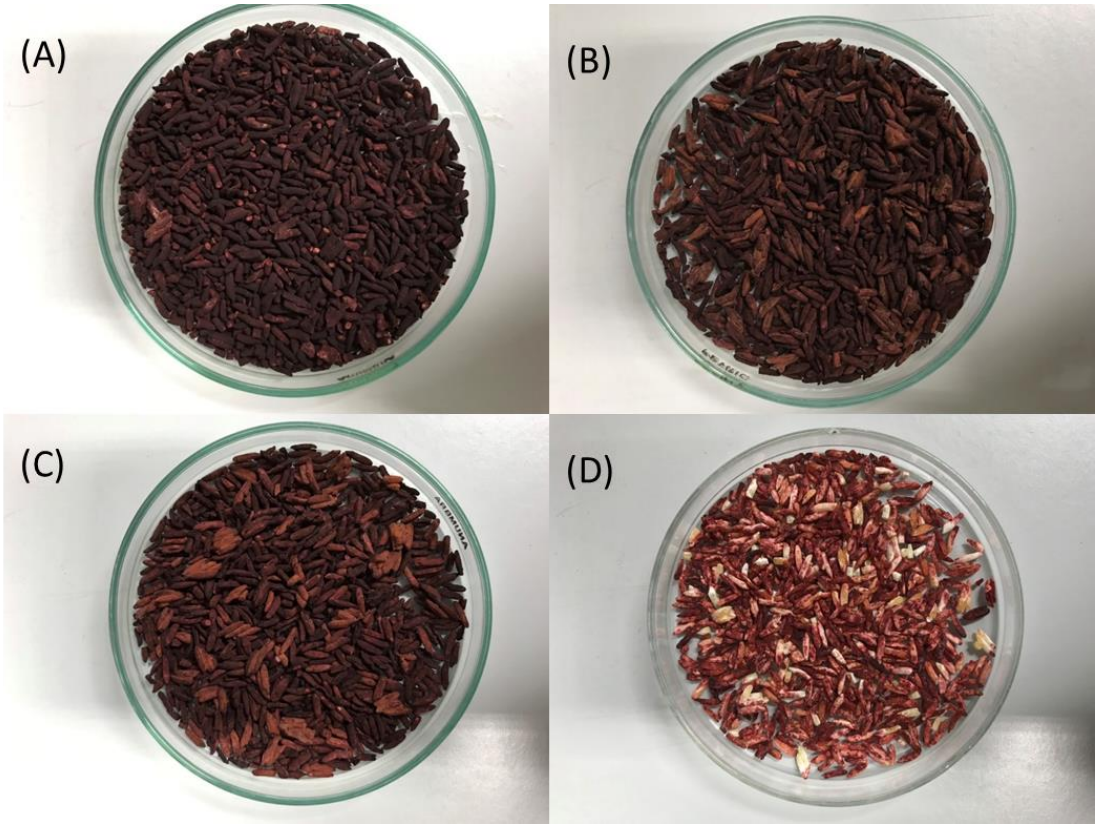

Fig. 2-3. Red koji after 7 days cultivation at 30°C by the wt strain KUPM5 (A) and mutant strains KS301U (B), KS104N, (C), and KS1003C (D). All red koji samples were dried at 60°C for 24 h.

Table 2-3. Enzyme activity and red pigment production of *Monascus* mutant strains.

***M. purpureus* strains**	**Enzyme activity (Unit/ml)**			**Red pigment (A_{500}/g)**
	α-amylase	**protease**	**lipase**	
KUPM5 (wt)	4.89±0.65^{bcd}	2.63±0.67^{f}	5.39±0.99^{g}	9.17±0.41^{b}
KS301U	4.51±0.65^{bcd}	3.38±0.30^{ef}	9.21±0.73^{f}	6.95±0.30^{c}
KS302U	4.26±0.22^{cd}	6.43±0.72^{d}	12.72±0.27^{e}	9.05±0.80^{b}
KS101N	6.52±0.95^{a}	15.07±0.40^{a}	13.25±1.87^{de}	5.37±0.24^{d}
KS102N	3.51±0.95^{d}	13.20±1.05^{b}	16.89±1.06^{bc}	7.00±0.31^{c}
KS103N	5.76±0.78^{ab}	14.28±0.89^{ab}	18.95±3.03^{ab}	10.03±0.48^{b}
KS104N	4.39±0.22^{cd}	13.60±1.00^{ab}	20.09±1.06^{a}	14.27±0.86^{a}
KS1001C	4.39±0.57^{cd}	4.78±0.47^{e}	14.30±1.80^{cde}	1.72±0.24^{e}
KS1002C	3.76±1.36^{cd}	8.93±0.33^{c}	15.48±1.67^{cd}	2.78±0.25^{e}
KS1003C	4.01±0.22^{cd}	8.66±1.76^{c}	15.83±0.42^{cd}	1.67±0.33^{e}
KS1004C	5.14±0.43^{bc}	9.32±1.27^{c}	15.70±0.55^{cd}	2.15±0.40^{e}

Different superscript letters (a, b, c, d, e, f, g) in the same column represent significantly different values ($p \leq 0.05$) and preparation of koji were carried out in triplicate.

2-4. Discussion

Red koji (red mold rice) is the product of *M. purpureus*, which has been widely used in the food industry. Citrinin, a secondary metabolite derived from the polyketide pathway of *M. purpureus*, is a known mycotoxin. Currently, there are concerns relating to citrinin contamination in red koji. Therefore, some researchers have studied methods to reduce citrinin production by *Monascus*. The most powerful technology to obtain the citrinin non-producing mutant is to rely on genetic engineering. In fact, the loss of function of *citS* involved in the first step of reaction of citrinin production leads to the complete elimination of citrinin production (81). However, in the field of food industry, classical mutagenesis using mutagens has been an effective technique to improve microorganisms. Wang *et al*. (82) have used UV irradiation as a physical mutagen and NTG as a chemical mutagen to induce the mutation of the *Monascus* strain to obtain citrinin non-producing strain. In this study, we used UV irradiation and NTG treatment as mutagens to develop *Monascus* mutant strains with low citrinin production and screened them using plate bioassay. Citrinin production in the red koji produced by strains KS301U, KS302U, KS1002C, and KS1004C were 80%–100% less than that in the parental strain. However, strains KS101N, KS102N, KS103N, and KS104N produced higher amounts of citrinin than the parental strain, which conflicted with the results of our plate bioassay. This might be due to the difference in culture conditions between plate bioassay and red koji production (88, 89).

In this study, we used the *M. purpureus* strain KUPM5 isolated from Thai fermented food, *sufu*. Although *Monascus* strains that have been previously mutagenized are different from our *Monascus* strain, the present results are in agreement with those of previous researches that UV irradiation efficiently induced mutation within genes related to citrinin production but not within other genes, such as those related to red pigment production, enzyme activity, and fungi hyphal development (66, 82, 88)**.** Strains KS1001C, KS1002C, KS1003C, and KS1004C, selected after combined mutagenesis of UV and NTG treatments, eliminated citrinin production and

simultaneously reduced red pigment production, leading to colorless koji (Fig. 2-3). A fungal regulator protein, LaeA, is an upstream key regulator for secondary metabolism and sporulation. Alteration of the *laeA* expression leads to simultaneous change in secondary metabolite production in *Monascus* (90, 91). It is possible that these mutants have a mutation within the regulatory gene that functions at the upper level of secondary metabolism and commonly regulates each secondary metabolism. In contrast, strains KS301U and KS302U obtained by UV irradiation showed reduced citrinin production in red koji but maintained the ability to form red pigment similar to the parental strain. It is likely that the mutation occurred only in genes involved in citrinin production. We are in the process of conducting a comparative genome analysis between the wt and mutant strains, which will allow us to identify the mutation point in mutant strains.

Reduced citrinin production while maintaining red pigment production in strains KS301U and KS302U make them favorable mutants to produce red koji. With regards to the Thai *sufu* production, α-amylase, protease, and lipase activities were closely associated with the quality of *sufu*, such as flavor, taste and texture (87). Strain KS302U has the possibility to produce another type of *sufu* because of its higher enzyme activities than the wt, whereas strain KS301U produces the same type of *sufu* as the wt because of similar enzyme activity. Further studies examining *sufu* production using *Monascus* mutants should be reported to confirm the effect of each mutant and determine the quality of *sufu*.

Chapter 3. Genome sequencing and *de novo* analysis of *Monascus purpureus* KUPM5

3-1. Introduction

Filamentous fungus *Monascus* belongs to the family *Monascaceae*, the order *Eurotiales*, the subclass *Eurotiomycetidae*, and the class *Eurotiomycetes* (92). *Monascus* sp. was first identified by isolating from boiled potatoes in 1884. Only two species, *M. ruber* and *M. mucoroides* were identified. *M. purpureus* was later isolated from red rice (known as Angka). Since the year 1930, five species have been clearly studied and isolated including; *M. purpureus, M. barkeri Dangeard, M. olei Piedallu, M. mucoroides* and *M. rubber* van Tieghem (6).

Presently, *Monascus* spp. are classified based on physiological and morphological properties such as growth rate on solid media, pigment production and characteristics of ascocarp, and enzymatic properties. It can be classified into 25 species, namely *M. albidus, M. albus, M. anka, M. araneosus, M. barkeri, M. bisporus, M. floridanus, M. fuliginosus, M. kaoliang, M. major, M. mucoroides, M. olei, M. paxii, M. pallens, M. pilosus, M. pubigerus, M. purpureus, M. purpurescens, M. rubber, M. rubiginosus, M. rubropunctatus, M. sanguineus, M. serorubercens, M. vini* and *M. vitreus* (93). But only nine species are internationally accepted to use in the food industry including; *M. argentinensis, M. eremophilus, M. floridanus, M. lunisporas, M. pallens, M. pilosus, M. purpureus, M. ruber,* and *M. sanguineus* (94).

Red koji contained a variety of beneficial compounds, including pigments (natural food colorants), monacolins (anti-hypercholesterolemic agents), γ-aminobutyric acid (GABA, a type of hypotensive agent), dimerumic acid (a natural antioxidant), sterols, isoflavones, unsaturated fatty acids, protease and esterase, and it also has potential therapeutic action in several chronic diseases, including cancer prevention (95, 96). Therefore, it is become a widely consumed supplement worldwide. In recent years, several genes involved in the biosynthesis of citrinin, monacolin K

(MK), and pigments have been cloned and analyzed (33, 97, 98). It represents a significant step forward in our understanding of the secondary metabolism of *Monascus* species.

This chapter describes the genome analysis of *M. purpureus* strain KUPM5 and also identification of the gene clusters involved in citrinin and *Monascus* pigments. This genome information will provide insight into the molecular characteristics of *M. purpureus* KUPM5.

3-2. Materials and methods

3-2-1. Fungal strains, culture media, and growth conditions

M. purpureus strain KUPM5 was obtained from Faculty of Agro-industry, Kasetsart University. Strains was cultivated on potato dextrose agar (PDA) at 30 ℃ for 7 days. To prepare the mycelia for genomic analysis, spore suspensions were prepared and inoculated into potato dextrose (PD) liquid medium and cultivated at 30℃ for 7 days.

3-2-2. DNA extraction and sequencing

Mycelia of *M. purpureus* strains KUPM5 and mutants were harvested after growth on PDB medium at 30℃ for 7 days, washed with sterilized water, lyophilized, and kept at −80℃ until use. DNA was extracted with the ISOPLANT II kit (Nippon Gene, Japan) according to the manufacturer's instructions. The RNA contamination was eliminated by incubating with RNase A at 37℃ for 30 min. A paired-end short-read DNA library of all samples was prepared using the NEBNext Ultra DNA Library Prep Kit (New England Biolabs, UK) and sequenced using the Illumina Novaseq 6000 platform at Novogene Cooperation (China).

For the long-read sequencing of the genome of *M. purpureus* strain KUPM5 by a nanopore MinION, DNA was fragmented by Covaris g-TUBE (Covaris Inc., USA) at 9400 rpm for 60 s. The DNA library was prepared using the Genomic DNA by Ligation kit SQK-LSK109 (Oxford Nanopore Technologies, UK). The NEBNext FFPE DNA Repair Mix and dA-tailing Module reagents (New England Biolabs, UK) were used for DNA repair. For the clean-up step, the AMPure XP bead system (Beckman-Coulter, USA) was utilized according to the manufacturer's instructions. Then, the prepared library was loaded to the flow cell (FLO-MIN106D R9 version, MIN-101B) and the sequencing was performed with the MinKNOW software (version 4.2.8).

3-2-3. *De novo* assembly and analysis

The whole genome of the *M. purpureus* strain KUPM5 was assembled using NECAT (www.github.com/xiaochuanle/NECAT), Flye (www.github.com/fenderglass/Flye), SMART-denovo (www.github.com/ruanjue/smartdenovo) and CANU (github.com/marbl/canu) to obtain the best draft genome contig results, that were evaluated by QUAST v5.0.2 (99). Errors in genome contigs were corrected and polished by Medaka v.1.3.2 (www.github.com/nanoporetech/medaka). The adapters and low-quality Illumina short-read data sequences were trimmed using Trimmomatic v.0.39 (100). Trimmed reads were mapped to polished genome contigs using Burrows–Wheeler Aligner v0.7.17 (101), and the mapped data were sorted and converted to bam files using SAMtools v1.12 (102). Further polishing was performed four times by Pilon v1.24 (103) using the mapping data.

3-2-4. Gene prediction and annotation

Annotation of the assembled genome was performed using Funannotate pipeline v1.8.3 (www.github.com/nextgenusfs/funannotate) to predict coding genes. Secondary metabolite biosynthetic gene clusters (BGCs) were annotated using antiSMASH fungus version 6.0 (104) to predict a putative secondary metabolite biosynthesis in *M. purpureus* KUPM5.

3-2-5. Nucleotide sequence accession numbers

Read data are available at the DDBJ Sequence Read Archive, under accession numbers DRA012480 and DRA012595.

3-3. Results

3-3-1. Overview of whole genome sequencing and assembling

The whole genome sequence of *M. purpureus* KUPM5 was performed using 4 assemblers including; NECAT, Flye, SMARTdenovo, and CANU. The overall genome sizes were 24.2, 24.3, 24.4, and 20.5 Mb, respectively. QUAST computed the number of contigs. Each assembler approach could produce the smallest number of contigs that match the whole genome sequence. CANU produced the highest contigs, followed by Flye, whereas SMARTdenovo and NECAT produced the lowest contigs by 10 and 11, respectively. The N50 contig length was determined by running the QUAST script on contig files generated by those four assemblers. SMARTdenovo, Flye, and NECAT showed a high N50 contig lengths of 3.07, 3.06, and 2.51 Mb, respectively, whereas CANU produced the lowest N50 contig length of 0.5 Mb. The percentage of GC content by four assemblers was similar at approximately 48% (Table 3-1).

Table 3-1. Comparison of draft *M. purpureus* KUPM5 genome general features made by four assemblers.

Statistics	**Assemblers**			
	NECAT	**Flye**	**SMARTdenovo**	**CANU**
contigs	11	20	10	77
Total length (bp)	24,191,400	24,284,000	24,430,026	20,510,783
N50 (bp)	2,509,075	3,066,537	3,070,228	538,382
%GC	48.84	48.92	48.89	48.5

The SMARTdenovo assembler was chosen to generate the high-quality whole genome sequence of strain KUPM5 because it produced the fewest number scaffold as 10 scaffolds (Table 3-2), that were close to the 8 chromosomes of *Monascus* spp. (105). The draft dataset created by SMARTdenovo was then corrected and polished using Medaka v.1.3.2 and Pilon v1.24. Four rounds of pilon polishing were performed to improve assembly quality. The size of genome sequence was improved to 24.47 Mb with 3.075 Mb of N50 and the GC content was improved rather to 48.92% (Table 3-3).

Funannotate generated 9,270 predicted genes with 10,056 CDS and 119 tRNA. To classify the functions of the coding genes, all coding sequences (CDSs) were subjected to COG analysis. The COG database (http://www.ncbi.nlm.nih.gov/COG) classifies proteins by comparing all protein sequences in the genome (106). In total, 10,056 CDSs were allocated to COG categories (Table 3-4), with the maximum proportion of sequences related to "Metabolism" (30.48%), followed by "Cellular Processes and Signaling" (18.38%) and "Information Storage and Processing" (17.67%). Proteins that have not been fully identified in the genome of strain KUPM5 were classified as "function unknown" (27.79%).

Table 3-2. The assembly result of *M. purpureus* KUPM5 produced by SMARTdenovo assembler.

Number of scaffolds	Length (bp)	%GC
Scaffold 1	4,544,634	49.04
Scaffold 2	3,413,253	48.77
Scaffold 3	3,294,240	48.89
Scaffold 4	3,075,987	48.84
Scaffold 5	2,996,661	49.14
Scaffold 6	2,747,800	48.99
Scaffold 7	2,308,511	48.74
Scaffold 8	2,050,087	48.81
Scaffold 9	25,647	57.46
Scaffold 10	19,165	47.02

Table 3-3. Comparison of the improved assembly genome of *M. purpureus* KUPM5 between the first and final rounds of the Pilon strategy.

Statistics	Pilon polishing	
	1st round	Final round (4th)
contigs	10	10
Total length (bp)	24,430,026	24,475,985
N50 (bp)	3,070,228	3,075,987
%GC	48.89	48.92

Table 3-4. Clusters of Orthologous Groups categories distribution of *M. purpureus* KUPM5.

Information Storage and Processing	
Translation, ribosomal structure and biogenesis (J)	389
Transcription (K)	388
RNA processing and modification (A)	342
Replication, recombination and repair (L)	270
Chromatin structure and dynamics (B)	77
Total	1,466/17.67%
Cellular Processes and Signaling:	
Posttranslational modification, protein turnover, chaperones (O)	460
Intracellular trafficking, secretion, and vesicular transport (U)	431
Signal transduction mechanisms (T)	309
Cytoskeleton (Z)	107
Cell cycle control, cell division, chromosome partitioning (D)	100
Cell wall/membrane/envelope biogenesis (M)	59
Defense mechanisms (V)	47
Extracellular structures (W)	5
Nuclear structure (Y)	4
Cell motility (N)	3
Total	1,525/18.38%
Metabolism:	
Amino acid transport and metabolism (E)	493
Carbohydrate transport and metabolism (G)	474
Secondary metabolites biosynthesis, transport and catabolism (Q)	345
Energy production and conversion (C)	332
Lipid transport and metabolism (I)	296
Inorganic ion transport and metabolism (P)	246
Coenzyme transport and metabolism (H)	234
Nucleotide transport and metabolism (F)	109
Total	2,529/30.48%
Poorly Characterized:	
Function unknown (S)	2306
General function prediction only (R)	0
Total	2,306/27.79%

3-3-2. Identification of secondary metabolites biosynthetic gene clusters (BGCs)

AntiSMASH is a powerful tool for identifying and annotating secondary metabolite biosynthetic gene clusters (BGCs) in bacterial, plant, and fungal genomic sequences. To better understand the production of secondary metabolites in *M. purpureus* KUPM5, a putative BGCs were predicted using antiSMASH fungi version 6.0. There are a total of 23 biosynthetic gene clusters (BGCs) predicted including; Type I polyketide (T1PKS), non-ribosomal peptide synthase (NRPS), NRPS-like, T1PKS/NRPS hybrid, betalactone, and terpene (TE), and 7 known BGCs and the other 16 putative gene clusters (Table 3-5).

Following the database search using antiSMASH, a BGC for citrinin was predicted within the genome sequence of strain KUPM5, which was identical to the known citrinin BGC of *M. ruber* M7 (GenBank accession number KT781075.1), and the identity of homologous genes was 62%. Furthermore, 12 homologous genes were identified, including *citA*, *citB*, *citC*, *citD*, *citE*, *citS*, *mrl5*, *mrr1*, *mrr2*, *mrr3*, *mrr4* and *mrr5* (Fig. 3-1A).

In the genome of strain KUPM5, a potential BGC responsible for *Monascus* pigments (MPs) production was found with 29% of homologous genes showing similarity to the database (GenBank accession number KC148521.1) (Fig. 3-1B). In strain KUPM5, only six similar genes were found: *MpigA*, *MpigD*, *MpigE*, *MpigF*, *MpigG* and *MpigI*. Furthermore, no complete BGC of monacolin K was found in the genome of *M. purpureus* strain KUPM5.

Table 3-5. The distribution of biosynthetic gene clusters in *M. purpureus* KUPM5.

Region	Gene types	Location (nucleotides)		Most similar known cluster	Similarity
		From	To		
Scaffold 1	T1PKS, NRPS	69,134	108,861	-	
	NRPS	345,174	392,480	-	
	NRPS, T1PKS	3,843,545	3,914,248	-	
	T1PKS	4,402,354	4,449,760	-	
Scaffold 2	NRPS	1,205,929	1,247,843	-	
	Betalactone	2,524,764	2,553,591	-	
	NRPS-like	2,687,361	2,730,675	-	
	NRPS	3,235,011	3,281,524	-	
Scaffold 3	NRPS	1,309,716	1,367,109	-	
	NRPS-like	1,784,152	1,829,014	-	
	Terpene	2,570,099	2,594,944	-	
	T1PKS	3,182,760	3,226,308	Tetrahydroxynaphthalene	100%
Scaffold 4	NRPS	2,370,234	2,414,080	-	
Scaffold 5	Terpene	120,046	141,457	Squalestatin S1	60%
	NRPS, T1PKS	1,405,637	1,457,474	NG-391	50%
	NRPS-like	1,820,165	1,863,848	-	
	T1PKS	2,547,149	2,593,407	Citrinin	62%
	Terpene	2,910,872	2,932,273	Aspterric acid	100%
Scaffold 6	NRPS-like	1,612,903	1,656,724	-	
	T1PKS	2,315,634	2,363,918	Ankaflavin, monascin, rubropunctatine, monascorubrin	29%
Scaffold 7	Betalactone	2,096,533	2,120,585	-	
Scaffold 8	T1PKS	148,510	197,188	Cornexistin	14%
	NRPS-like	1,718,488	1,760,985	-	

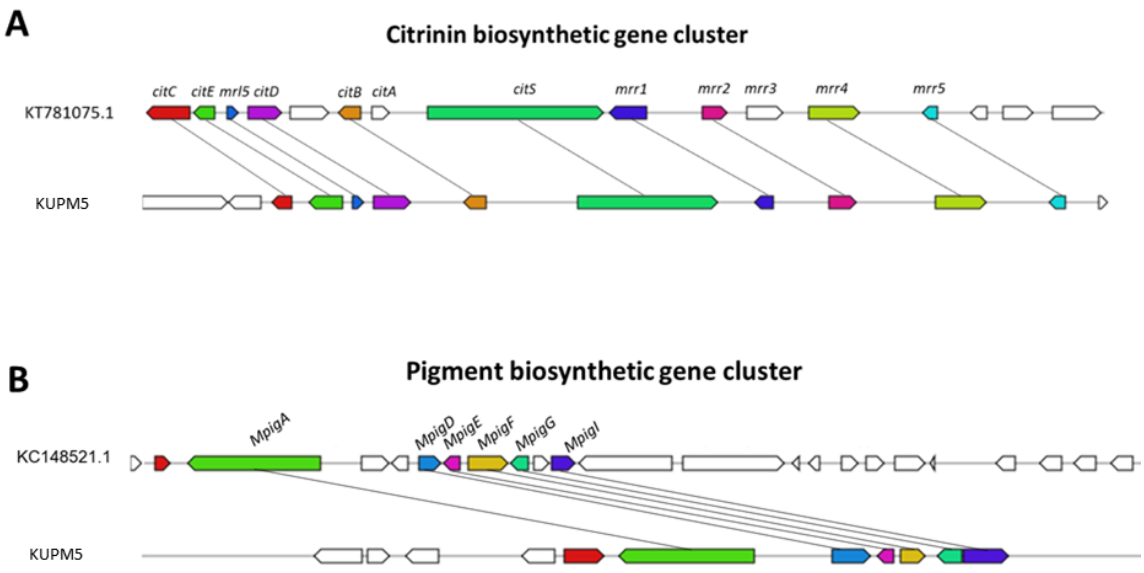

Fig. 3-1. Secondary metabolites BGCs in the genome sequence of *M. purpureus* KUPM5.

3-4. Discussion

We obtained the whole-genome information of *M. purpureus* KUPM5 in this work, which will be highly beneficial for future studies on chromosomal evolution and genetic diversity in *Monascus*, as well as a significant resource for biological research and industrial applications. With the ongoing improvement of sequencing technology and bioinformatics, major progress has been made in the study on biosynthesis pathways of secondary metabolites, the most notable of which are *Monascus* pigments, citrinin, and monacolin K. In this study, several assemblers including NECAT, SMARTdenovo, Flye and CANU were used to generated draft genome. Only SMARTdenovo generated the fewest number scaffold as 10 scaffolds. The SMARTdenovo assembler includes a variety of methods that have not been implemented in other programs, such as its weighting strategy for controlling repetition regions, which substantially improves its speed and alignment precision (107). Therefore, SMARTdenovo was chosen as an appropriate assembler for *M. purpureus* KUPM5.

The COG analysis of 10,056 CDSs in *M. purpureus* KUPM5 was categorized into 25 groups. The biosynthesis of secondary metabolites (Q) group was the most interesting because it involves secondary metabolite synthesis. The study revealed that *M. purpureus* KUPM5 contained only 345 unigenes, which was lesser than other fungi such as *Fusarium* sp. R1 (108), *A. flavus* (109), and *P. digitatum* (110) by approximately 800, 900, and 500 unigenes, respectively. These findings indicate that the number of unigenes in Q group is directly related to the number of BGCs, where *M. purpureus* KUPM5 had only 23 BGCs compared to other fungi such as *Fusarium* sp. R1 which had 37 BGCs (108).

In 2005, the citrinin biosynthetic key gene PKS was discovered in *M. purpureus* (32). Type I PKSs (modular type I PKSs and iterative type I PKSs), type II PKSs, and type III PKSs are the most common forms of microbial PKSs. Citrinin biosynthesis is mediated by an iterative type I PKS that comprises putative domains for ketosynthase (KS), acyltransferase (AT), ketoreductase (KR), dehydratase (DH), enoyl reductase (ER), methyltransferase (MT), thioesterase (TE), and acyl carrier protein (ACP) (32). The KS domain catalyzes the condensation of precursors to lengthen the polyketone chain, while the AT domain chooses the precursors and the ACP domain forms covalent connections between the precursors and intermediates, which are required for most PKSs to operate (111). The citrinin BGC of *Monascus aurantiacus*, with a length of 43 kb, was initially reported in 2012 (112), encompassing 16 open reading frames (ORFs) *for ctnD, ctnE, orf6, orf1, ctnA, orf3, orf4, pksCT, orf5, ctnF, orf7, ctnR, orf8, ctnG*, some of which are similar to those of the citrinin BGC of *M. purpureus* strain KUPM5. These findings demonstrated a significant similarity of citrinin BGC in *Monascus*, particularly the essential gene PKS. A putative 53 kb pigments BGC of *M. ruber* was originally identified in 2012, and it included genes encoding PKSs, fatty acid synthases, regulatory factors, and dehydrogenase (113). *M. purpureus* genome was discovered to be smaller than that of related filamentous fungus, indicating a considerable loss of genes (105). Following the prediction of MK BGC in the genome of strain KUPM5, we discovered that there was no MK BGC in the strain KUPM5, but only a subset of genes appeared nearby. Identifying BGCs has undoubtedly improved knowledge of the biosynthetic pathways of secondary metabolites in *Monascus*, which can give theoretical basis for industrial production of *Monascus* secondary metabolites.

Chapter 4. Development of *Monascus purpureus* monacolin K hyperproducing mutant strains by synchrotron light irradiation and comparative genome analysis

4-1. Introduction

Since the discovery of *Monascus* sp. by Van Tieghem in 1884, various *Monascus* products have attracted attention worldwide. *Monascus* spp. play a significant role in household food consumption and industrial food manufacturing, producing rice-fermented products, natural colorants, natural foodadditives, pharmaceuticals, and dietary supplements (114). Especially in Asian countries, *Monascus* spp. were also known as a traditional expediently filamentous fungus because of the utilization of their substances. In addition, certain species can produce substantial secondary metabolites, such as *Monascus* pigments, dimeric acid, γ-aminobutyric acid, and monacolin K (MK); some strains, such as *M. purpureus* and *M. ruber*, also produce a harmful aromatic polyketide substance known as citrinin, which is nephrotoxic (76, 115).

MK is one of statin analog, is a famous drug which has been applied widely because of its pharmacological hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, the key enzyme in cholesterol synthesis, and terminating mevalonate synthesis, which results in obesity prevention; other effects, such as anti-inflammatory and anticancer effects, have also been proven (116, 117). Furthermore, MK was confirmed to be highly efficacious in treating neurological disorders, such as Parkinson's disease, depression, and schizophrenia (118, 119). MK consists of two polyketides probably produced by two distinct polyketide synthases starting with acetyl-CoA and malonyl-CoA as substrates (120). Lovastatin (a synonym of MK) biosynthetic gene clusters were first discovered in *Aspergillus terreus* containing nine genes (121). The MK gene cluster was found originally in *Monascus pilosus* and resembles that of *A. terreus* in terms of the orientation of the corresponding genes (122). MK biosynthetic gene clusters include *mokA* and *mokB* that code for polyketide synthase, *mokC* for P450-monooxygenase, *mokD* for oxidoreductase, *mokE* for dehydrogenase,

mokF for transferase, *mokG* for HMG-CoA reductase, *mokH* for a transcription factor, and *mokI* for an efflux pump (122-124).

Induced mutation technology has undergone numerous improvements to develop the constant need for ideal strains. There are various potential strategies such as stress-induced mutations (125), physical and chemical-induced mutations (63), or molecular genetic adaptive mutations (126). Although genetic modification is an excellent tool that is fast, accurate, and can directly adapt some gene functions, it is not suitable for consumers because of the potential risks involved. There is an ongoing debate in health effects among producers, consumers, and policymakers (127). In a previous study (128), we induced the mutation in *M. purpureus* strain KUPM5 by ultraviolet (UV) irradiation and nitrosoguanidine (NTG) treatments to improve the strains showing the reduced production of a mycotoxin, citrinin. Those mutagens were proven to be effective in reducing the productivity of citrinin. Synchrotron radiation accumulates accelerated high energy in a storage ring and is a powerful physical mutagen (45). Recently, Baba *et al*. successfully obtained various mutants of *Saccharomyces cerevisiae* by synchrotron light irradiation and revealed the characteristics of mutations in the sake yeast strain (129). An important issue regarding mutations by synchrotron radiation is insufficient research, especially in filamentous fungi. Liang *et al*. reported that mutations induced by 0.54-keV X-rays of synchrotron light demonstrated high efficiency in the strain improvement of *Aspergillus oryzae*, which increased kojic acid productivity by 56% (130). However, no research on *Monascus* species has been attempted.

In this study, we induced mutations in *M. purpureus* KUPM5 by synchrotron light irradiation to obtain MK-hyperproducing strains. Then, we performed comparative genomic analysis of selected mutants and the parental strains, to detect the various mutations in the genome. Thus, synchrotron light-induced mutation has been a powerful alternative tool for developing new strains in *M. purpureus*.

4-2. Materials and methods

4-2-1. Microbial strains and culture conditions

M. purpureus strain KUPM5 was used as the parental strain for the introduction of mutations (128). The strain KUPM5 was cultured on potato dextrose agar (PDA) medium (potato starch 4 g, dextrose 20 g, and agar 15–20 g/l of water) at 30℃ for 7 days. The *Monascus* spore suspension was prepared by adding 0.01% Tween-80 to *Monascus* growth plates. The number of spores was counted and adjusted to 10^6/ml. *S. cerevisiae* strain K7 was cultivated in yeast extract peptone dextrose (YPD) liquid medium (yeast extract 10 g, peptone 20 g, and dextrose 20 g/l of water) and used for a plate bioassay to detect MK production.

4-2-2. Fungal mutagenesis by synchrotron light irradiation

A *Monascus* spore suspension (10^6 spores/ml) was placed in a 5-mL polystyrene tube and fixed in the chamber for synchrotron light irradiation. The spore suspensions were irradiated at three absorbed doses of ionizing radiation, 50, 100, and 300 Gray units (Gy), adjusted by the thickness of aluminum sheets at 0, 0.52, and 0.30 mm, with a 1.4 GeV proton beam and 250 mA (8-μm diameter beam size) obtained from Kyushu Synchrotron Light Research Center, Japan. Those suspensions not irradiated were used as controls. Irradiated samples were washed three times with sterilized water, then inoculated into PDB medium (potato starch 4 g and dextrose 20 g/l of water) and maintained in the dark at 30℃. After 6 h, spores were spread on PDA medium with 0.01% Triton X-100 and incubated at 30℃ for 72 h. The colonies developed were further used for plate bioassays. The rate of cell survival was calculated to determine the optimal irradiation condition.

4-2-3. Plate bioassays for MK-hyperproducing strains

Screening of the mutant strains with MK-hyperproducing ability was performed according to the method described by Ferrón *et al*. (131), with a slight modification. *S. cerevisiae* strain K7 was resuspended in a fresh YPD medium and incubated at 30℃ for 24 h. After adding 100 µl of the culture to YPD soft agar medium (0.7% agar), it was immediately poured into the agar medium on which the *Monascus* colonies were growing and incubated for 72 h. After 7 h of incubation at 30℃, the inhibition zone that appeared around the *Monascus* colonies was measured. MK-hyperproducing mutants were selected by their larger diameter than the parental strain KUPM5.

4-2-4. Making of *Monascus* red koji

Monascus koji, termed red koji, was produced according to our previous study (128), with a slight modification. Thai long-grain rice (*Oryza sativa*) was soaked in water for 4 h and then added to a 100-ml Erlenmeyer flask (7.0 g). Three milliliters of distilled water was added and autoclaved at 121℃ for 20 min. After the steamed rice was cooled, 1 ml of the *Monascus* spore suspension (10^6/ml) was inoculated, mixed well, and incubated at 30℃. Koji was made using each *Monascus* strain in triplicate.

4-2-5. Analyses of secondary metabolites

Red koji was dried at 55℃ for 24 h. The dried koji was powdered using grinding equipment, the Multibeads shocker MB601U (Yasui Kikai, Japan), with shaking at 1,500 rpm for 60 s. Two milliliters of 70% (v/v) ethanol was added to the tube containing 0.5 g red koji powder and incubated at 55℃ for 1 h. Then, the supernatants were collected by centrifugation and filtered through a 0.45-µm cellulose acetate membrane (ADVANTEC, Japan). MK was separated by HPLC

using a C18 column (TSKgel ODS 4.6 mm × 250 mm, Tosho, Japan). HPLC was performed with a mobile phase, consisting of 80% acetonitrile:0.1% phosphoric acid (60:40), column temperature at 30℃, flow rate of 1 ml/min, and detection at a wavelength of 238 nm. The amounts of citrinin were determined by the method described previously (128).

Red (monascorubramine and rubropunctamine), yellow (monascin and ankaflavin), and orange (monascorubrin and rubropunctatin) pigments from red koji were extracted according to the method of Dikshit and Tallapragda (132). The dried powder koji (1.0 g) was extracted with 10 ml of methanol. This mixture was incubated with shaking at 200 rpm for 24 h. After filtration through a 0.45-μm cellulose acetate membrane, the content of the pigment compounds was determined using a UV–visible spectrophotometer at 510, 480, and 400 nm to detect red, orange, and yellow pigments, respectively. One unit of the pigment yield was defined as showing 1 absorbance value at wavelengths of 510, 480, and 400 nm.

4-2-6. Analyses of α-amylase activity in red koji

The crude enzyme from red koji was prepared, and α-amylase activity was determined according to our previous study (128). Briefly, a substrate solution (3.9 ml) containing 1% gelatinized starch in 0.1 M McIlvaine buffer, pH 4.0, was preincubated for 10 min at 50℃. Then, the crude enzyme solution (0.1 ml) was added to the substrate solution to initiate the reaction and incubated for 10 min. Then, 0.2 ml of the reaction mixture was added to 5 ml of 0.167 mM iodine solution. The absorbance at 770 nm was used to determine the α-amylase activity. One unit of α-amylase was defined as the amount of absorbance that changes by 1 per min.

4-2-7. Fungal growth in red koji

The fungal mycelial weight corresponding to fungal growth in red koji was estimated by using the contents of *N*-acetylglucosamine (GlcNAc), which constitutes chitin, a cell wall component of *Ascomycetes* (133). The dried koji powder (0.5 g) was washed three times with 50 mM sodium phosphate buffer, pH 7.0 by centrifugation at 10,000 × g, 4℃ for 5 min. The samples were then resuspended in 5 ml of 50 mM sodium phosphate buffer, pH 7.0, 10 mg of Yatalase was added (Takara Bio, Japan), and incubated at 37℃ for 60 min. The GlcNAc content was determined according to the method of Reissig *et al*. (134).

4-2-8. Statistical analysis

All experiments for determination of fungal metabolites and enzyme activity were performed in triplicate. The statistical analysis was done using Student's t-test with significantly differences between samples ($p = 0.05$).

4-2-9. Fungal genomic analyses

Experimental methods for genomic DNA extraction and *de novo* analysis of *M. purpureus* KUPM5 and the mutant strains are described in Chapter 3.

4-2-10. Variant calling for mutation pattern analysis

The reference genome was indexed with SAMtools v1.12 (135), creating BAM-index files. After post-processing step, we detected the genetic variants with the tools named Freebayes v1.3.5 (136). BCFtools (137) and RTG-Tools (138) were finally applied to extract the variant calls statistics.

4-3. Results

4-3-1. Induced mutation by synchrotron light irradiation

M. purpureus strain KUPM5 used in this study was obtained by isolation from Thai fermented food and has been used in our previous studies (72, 128). Synchrotron light-irradiated strain KUPM5 yielded mutants that exhibited higher MK production than the parent strain. Four absorbed doses of ionizing radiation were used to determine the optimal irradiation condition. The number of survivors correlates with the efficacy of mutations induced by synchrotron radiation, with a small number of survivors indicating a high frequency of mutations. The lowest survival rate of 3.90% was observed at 300 Gy, whereas survival rates of 21.67% and 61.1% were observed at doses of 100 and 50 Gy, respectively (Fig. 4-1). After mutagenesis, we selected *Monascus* mutants by synchrotron light irradiation at 300 Gy, followed by a plate bioassay using *S. cerevisiae* strain K7. Among 936 colonies formed on the PDA medium, three colonies termed strains SC01, SC02, and SC03 showed a larger inhibition zone than the parental strain KUPM5. Colonial characteristics of these mutants were observed over a range of 7-d growth on PDA plates (Fig. 4-2A). There were no differences among strains KUPM5, SC01, SC02, and SC03 with regard to the colony size after 3 days of cultivation on PDA medium. The colony size of strain SC03 was smaller than those of the other strains by 1 day of cultivation (Fig. 4-2B). The colony color of strain SC03 was irregular, ranging from light red to orange.

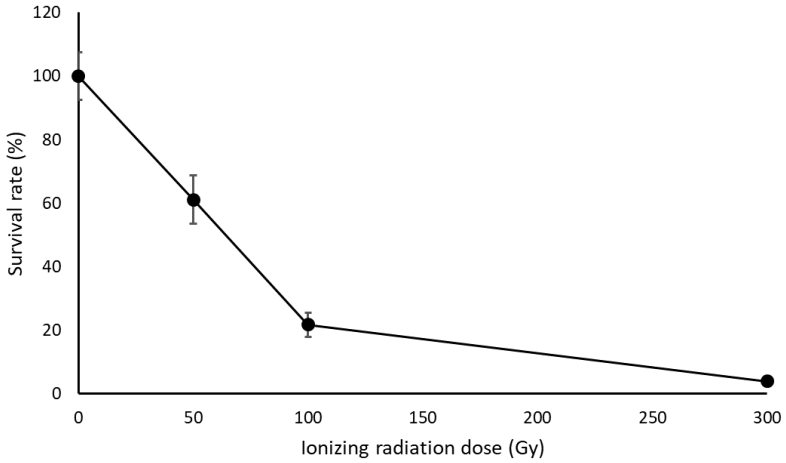

Fig. 4-1. The survival rate of *Monascus* spore suspension after synchrotron light irradiation treatment with different ionizing radiation dose at 0, 50, 100 and 300 Gy.

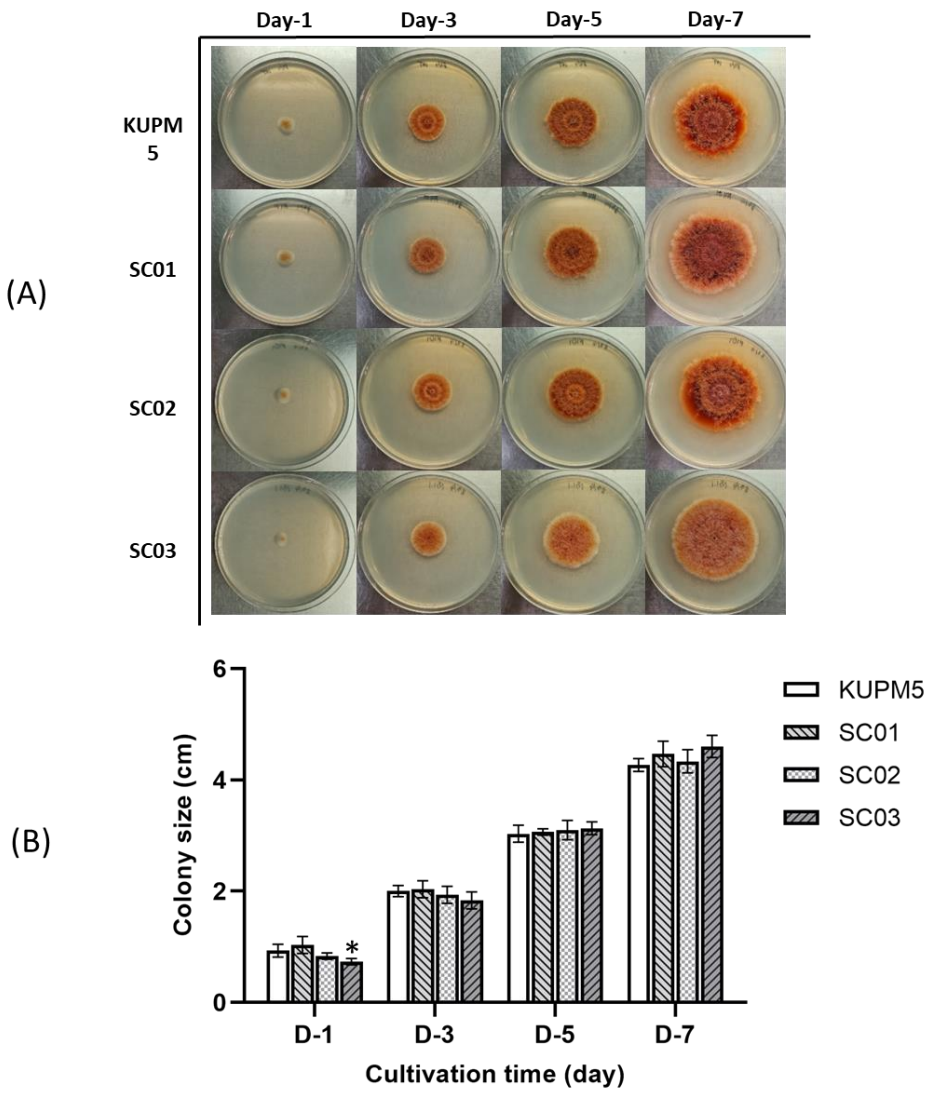

Fig. 4-2. (A) Colonial morphologies of *M. purpureus* growth and (B) colony size in a diameter (cm) of each mutant strains SC01, SC02, and SC03 compared with strain KUPM5 on PDA at 30℃ for 7 days. Error bars indicate the standard deviations in triplicate experiments. The "*" indicates a significant difference ($p < 0.05$) compared to the strain KUPM5 (white bar).

4-3-2. MK production in red koji

The selected mutant strains SC01, SC02, and SC03 were cultivated in rice to make red koji in a flask to determine the production of MK and other secondary metabolites. Red koji produced by the three mutant strains and parental strain KUPM5 were taken every 2 days until the 10th day of the cultivation period. Strains SC01, SC02, and SC03 produced a larger amount of MK than did the strain KUPM5 after the 8th day of cultivation (Fig. 4-3). In particular, strains SC02 and SC03 showed higher MK production, reaching up to 86.25±13.64 and 68.00±4.92 mg/g koji at 10 days of cultivation, which is approximately 3-fold and 2.5-fold higher than strain KUPM5 (26.08±1.83 mg/g), respectively.

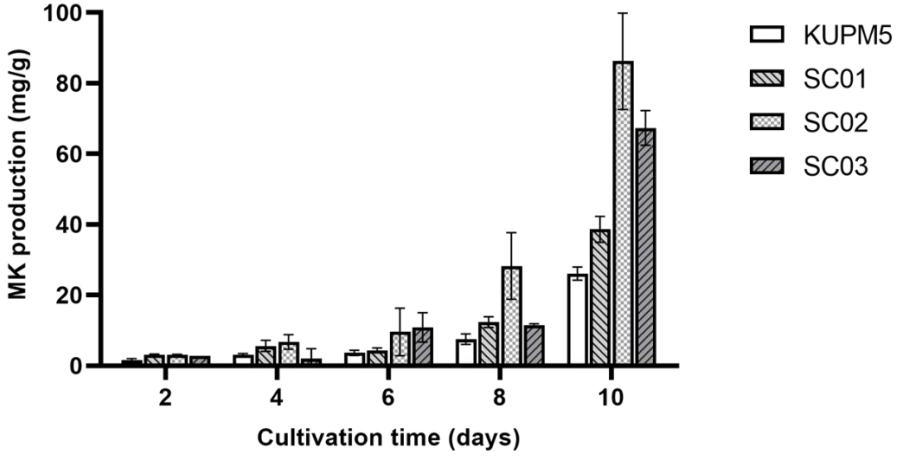

Fig. 4-3. Amounts of monacolin K in red koji made by mutant strains SC01, SC02, and SC03 compared to strain KUPM5. All samples were collected, and the production yields were determined using HPLC every 2 days. Error bars indicate the standard deviations in triplicate experiments. The "*" indicates a significant difference ($p \leq 0.05$) compared to the parental strain (white bar).

4-3-3. Pigments and citrinin production in red koji

Red, orange, and yellow pigments are also secondary metabolites produced by *Monascus*. The contents of the pigments in red koji that determine the color of koji were measured after 10 days of cultivation (Fig. 4-4A). Although the mutant strain SC01 tended to reduce the production of the three pigments, there was no significant difference between the SC01 and KUPM5 strains. The mutant strain SC02 produced red, orange and yellow pigments similar to strain KUPM5 at 16.27±2.45, 15.44±2.33, and 20.84±2.13 U/g koji, respectively. In contrast, all pigment contents produced by strain SC03 were reduced compared to those produced by the strain KUPM5, although this strain produced more MK than the parental strain KUPM5.

Citrinin is also a secondary metabolite produced by *Monascus*. As for citrinin production after 10 days of cultivation, strain SC01 showed citrinin productivity of 1.62 ± 0.08 ng/g koji similar to that of the wild type (2.02±1.04 ng/g), whereas strains SC02 and SC03 produced 5.09±0.11 and 5.70±0.50 ng citrinin/g koji, respectively, more than 2–2.5-fold higher than that of KUPM5 (Fig. 4-4B).

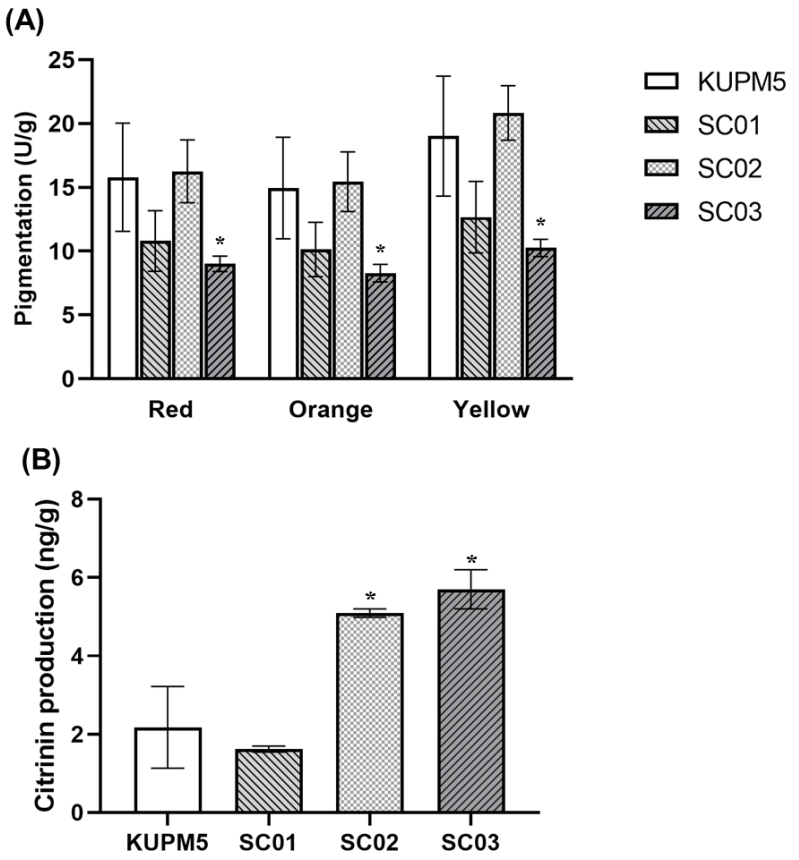

Fig. 4-4. Secondary metabolite production in red koji made by mutant strains SC01, SC02, and SC03 compared to KUPM5. (A) *Monascus* red, yellow, and orange pigments and (B) citrinin production. All experiments were determined after 10 days of cultivation in triplicate; the "*" indicates the significant difference ($p \leq 0.05$) compared to the parental strain (white bar).

4-3-4. Amylolytic enzyme activity in red koji and fungal growth

M. purpureus secreted α-amylase to degrade starch from rice to use glucose as a carbon source in koji. Thus, the α-amylase activity of the KUPM5 and mutant strains were compared. Red koji extracts from strains SC01, SC02, and SC03 showed slightly higher α-amylase activities (23.36±3.89, 19.9±0.49, and 20.87±1.61 U/ml, respectively) than that from strain KUPM5 (18.09±0.41 U/ml) (Fig. 4-5A).

Fungal growth in red koji was compared by using the GlcNAc content as an indicator. The GlcNAc contents from red koji made by strains SC01 and SC02 were similar to that by strain KUPM5, whereas the GlcNAc contents from strain SC03 were reduced to 50%, indicating that the growth of strain SC03 in the rice is reduced compared to that of strain KUPM5 (Fig. 4-5B). Though all mutant strains possess the ability to saccharify the rice, similar to strain KUPM5, strain SC03 showed reduced growth in red koji, indicating that the primary metabolism of the SC03 has some defects in mutant strain SC03.

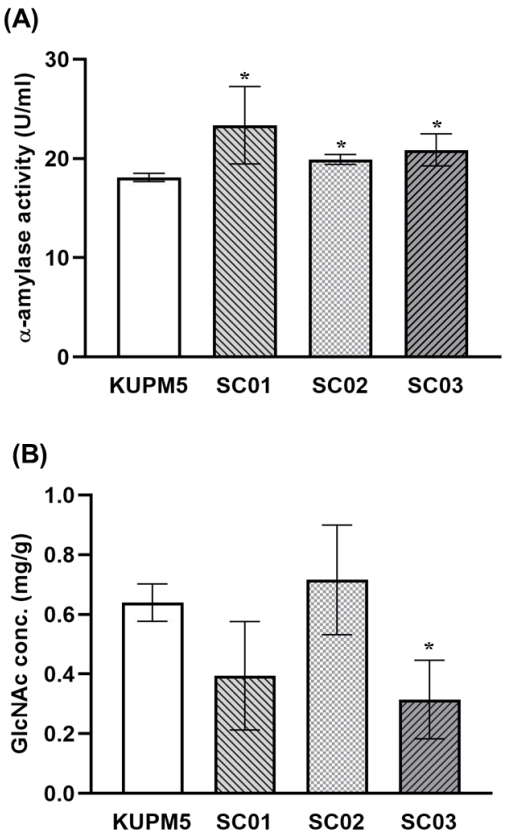

Fig. 4-5. (A) α-Amylase activity and (B) *N*-acetylglucosamine content. All experiments were performed in triplicate; the "*" indicates the significant difference ($p \leq 0.05$) compared to the parental strain (white bar).

4-3-5. Identification of monacolin K gene cluster of *M. purpureus*

The orthologous *mok* genes in strain KUPM5 were identified by using the *mok* gene (*mokA*–*mokI*) cluster of *M. pilosus* strain BCRC38072 (Genbank accession number DQ176595) as a query sequence (Fig. 4-6A). Strain KUPM5 carries entire *mok* genes distributed in the three separated scaffolds (Fig. 4-6B). All mutation sites were nucleotide substitutions. The amino acid substitutions within coding sequence (CDS) of *mok* orthologs was found as a missense mutation in *mokE* in strain SC03, resulting in change from proline at 84 amino acids to glutamine (Fig.4-7). In contrast, a number of nucleotide substitutions within 5'- and 3'-untranslated regions (UTR) that may affect the expression of *mok* genes were found in the *mok* gene in all mutant strains (Table 4-1). In strains SC01 and SC03, we found the mutation sites in the 5'- and 3'-UTRs of *mokA, mokB, mokD, mokF, mokG* and *mokI* orthologs. In strain SC01, a mutation of the 5'-UTR of *mokC* ortholog was additionally present. In strain SC02, we found mutation sites in the UTRs of *mokA, mokB, mokG* and *mokI* orthologs (Table 4-1). Although our method did not annotate the nucleotide sequence between *mokF* and *mokD,* we discovered the *mokH*-like region with mutation sites in the SC02 and SC03 mutants (Fig. 4-6B).

Then, we investigated *laeA*, coding for a master regulator that regulates the expression of several genes involved in secondary metabolite synthesis (139, 140). We found an ortholog of *laeA*, located in scaffold 2 with 100% amino acid identity to *M. purpureus* strain M1 (Genbank accession number QGA78456), and the mutation sites within *laeA* were also detected in three mutant strains (Fig. 4-6B).

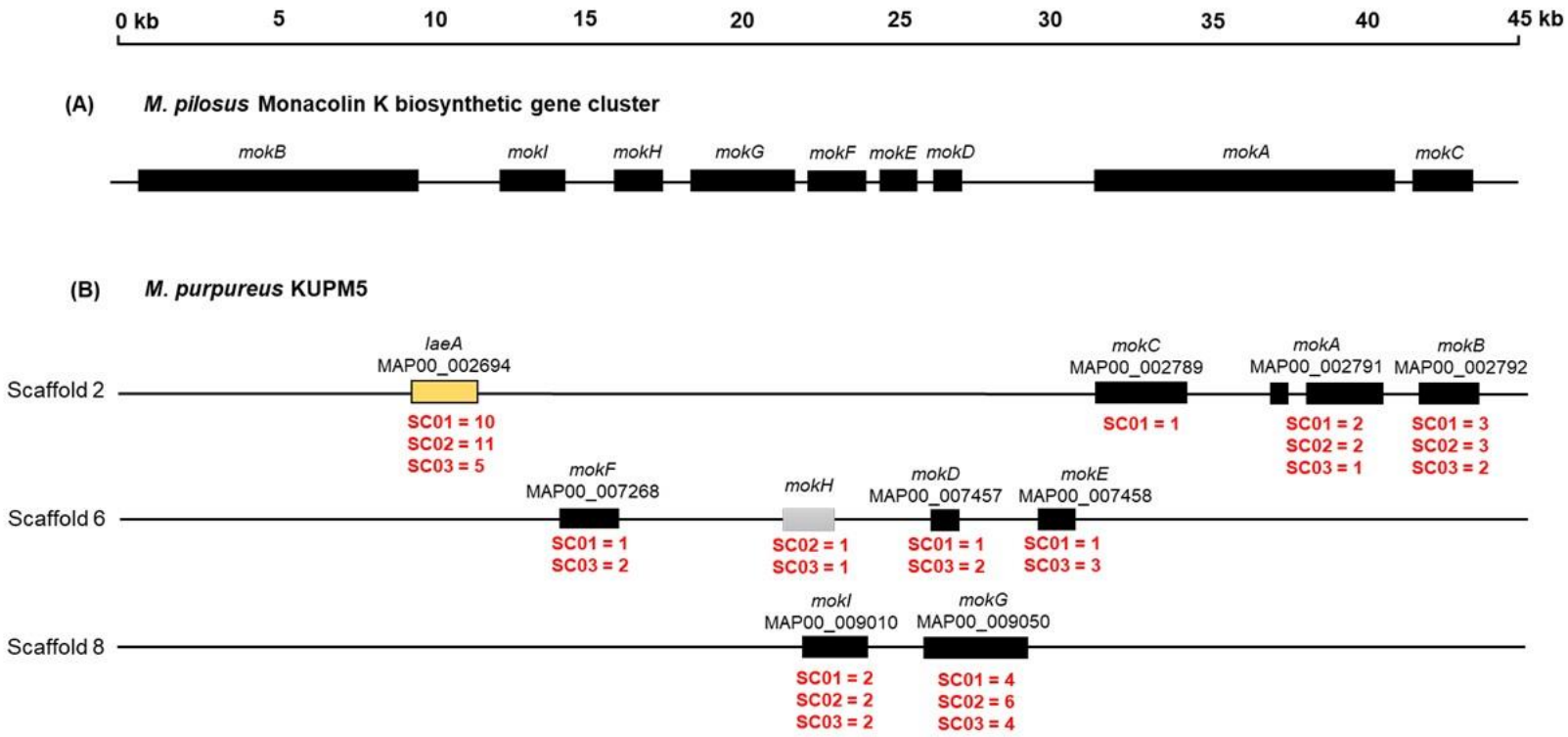

Fig. 4-6. Organization of *mok* gene orthologs of *M. purpureus* KUPM5 against the *mok* gene cluster of *M. pilosus* strain BCRC38072. (A) The *mok* gene orthologs were deposited in NCBI (GenBank Accession number DQ176595) at a length of 45 kb, (B) an identical region showing a homologous region to the MK gene cluster. The number in each gene indicates the mutated sites within the respective *mok* genes and the *laeA* gene in mutant strains SC01, SC02, and SC03. The size and length in each scaffold of (B) are not considered.

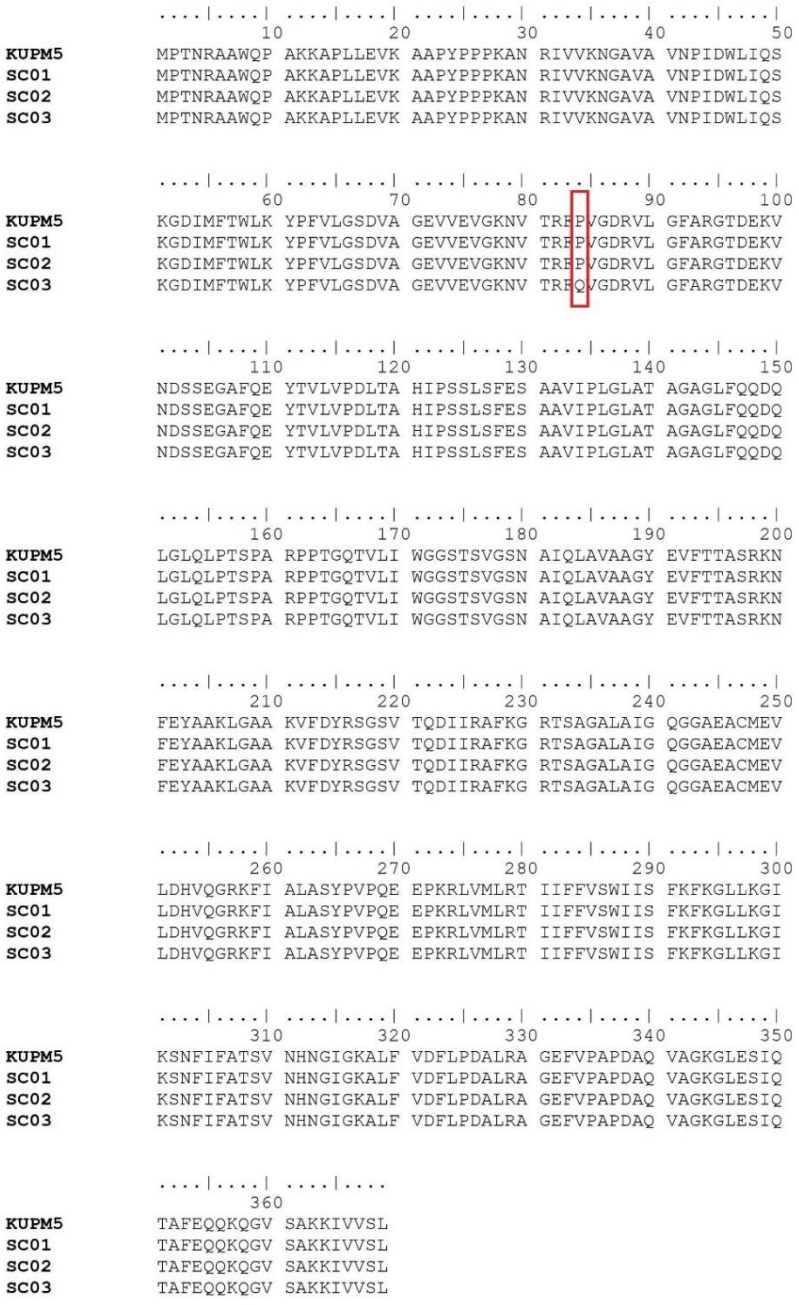

Fig. 4-7. Amino acid alignment of *mokE*-like between *M. purpureus* KUPM5 and mutants.

Table 4-1. Number of mutation sites with effect of sequence ontology among three mutant strain SC01, SC02 and SC03.

Annotated genes	Homologous genes	Functional variants annotation					
		SC01	No. of variants	SC02	No. of variants	SC03	No. of variants
MAP00_002791	*mokA*	3'-UTR*	1	3'-UTR*	2	3'-UTR*	1
		5'-UTR*	1				
MAP00_002792	*mokB*	3'-UTR*	2	3'-UTR*	3	3'-UTR*	2
		5'-UTR*	1				
MAP00_002789	*mokC*	5'-UTR*	1				
MAP00_007457	*mokD*	3'-UTR*	1			3'-UTR*	2
MAP00_007458	*mokE*	5'-UTR*	1			Missense**	1
						5'-UTR*	2
MAP00_007268	*mokF*	3'-UTR*	1			3'-UTR*	1
						5'-UTR*	1
MAP00_009050	*mokG*	5'-UTR*	1	5'-UTR*	1	5'-UTR*	1
		3'-UTR*	3	3'-UTR*	4	3'-UTR*	2
						5'-UTR*	1
N/A	*mokH*		0		1		1
MAP00_009010	*mokI*	5'-UTR*	2	5'-UTR*	2	5'-UTR*	2
MAP00_002694	*laeA*	5'-UTR*	7	5'-UTR*	8	3'-UTR*	4
		3'-UTR*	3	3'-UTR*	3	5'-UTR*	1
MAP00_001827	*mutT*	3'-UTR*	5	3'-UTR*	4	3'-UTR*	4
		5'-UTR*	2	5'-UTR*	1		
MAP00_008319	*mutY*	3'-UTR*	2	3'-UTR*	2	3'-UTR*	3
		Introns*	1	Introns*	1	Introns*	1
		5'-UTR*	2	5'-UTR*	2	5'-UTR*	2

* is 'MODIFIER' variant effect impact; regularly non-coding variants or variants affecting non-coding genes, which corresponding to gene expression, protein binding or transcription factor biding.

** is 'MODERATE' variant effect impact; a non-disruptive variation that may affect protein functionalities.

4-3-6. Mutation patterns induced by synchrotron irradiation

We examined the genetic variations in the whole genome of the parental strain KUPM5 and mutant strains SC01, SC02, and SC03 (Table 4-2). Types of mutations, including SNV, MNV, insertion and deletion (Indel), and multiple-nucleotide Indels, showed similar ratios among the three mutant strains. The average SNVs caused by synchrotron light mutation were about 68.96%; MNVs and multiple-nucleotide Indels exhibited the lowest values at 1.24% and 1.12%, respectively. Indel mutations reached up to 28.68% (insertion = 17.89% and deletion = 10.78%). As for SNVs, the number of base transversion substitution was approximately 92.38% higher than base transition changes with 7.62%. The highest transversion changes were of A:T to C:G in all mutant strains, whereas the least substituted bases were C:G to G:C. Besides, the G:C to A:T transitional substitution was the most frequently substituted base, followed by A:T to G:C (Table 4-3). The overall number of mutated genes including the 5′-untranslated region (UTR), CDS with introns, and 3′-UTR in the genomes of strains SC01, SC02, and SC03 were 8432, 8379 and 8295, respectively. However, the large number of gene variants makes it more difficult to identify the effect of each genetic variant. But it can be indicated that synchrotron light can induce a wide range of gene variations.

Table 4-2. Variants distribution in the mutant strains SC01, SC02, and SC03 by comparative genome analysis.

Type of variation	Ratio of types of variation (%)			Total variants (%)
	SC01	SC02	SC03	
SNP	69.10	69.34	68.43	68.96
MNP	1.18	1.28	1.28	1.24
Insertion	17.80	17.61	18.26	17.89
Deletion	10.84	10.58	10.92	10.78
Multiple Nucleotides and Indels	1.07	1.19	1.11	1.12

Table 4-3. The number of transversions and transitions that occurred in *M. purpureus* mutant strains SC01, SC02 and SC03.

Substitution types		Number of substitutions			
		SC01	SC02	SC03	Average
Transversion	A:T to C:G	3987	3953	3691	3877
	A:T to T:A	487	522	535	515
	G:C to T:A	922	956	919	932
	C:G to G:C	211	198	219	210
Transition	A:T to G:C	191	193	184	189
	G:C to A:T	271	268	262	267

Substitution types	Ratios of nucleotide substitution (%)			
	SC01	SC02	SC03	Average
Transversion	92.39	92.43	92.32	92.38
Transition	7.61	7.57	7.68	7.62

4-4. Discussion

Researchers concerned about the utilities of secondary metabolites in koji have tried to isolate the mutant strains exhibiting improved abilities to produce secondary metabolites. Using UV irradiation, *M. purpureus* mutant strain N310 was isolated, showing no citrinin production and maintaining MK production (82). In addition, *M. sanguineus* mutant strain UV-4 showing high lovastatin (MK) production and synthesizing a negligible amount of citrinin was isolated (132). However, another method, using synchrotron radiation as a physical mutagen, is also attracting attention and has been recognized as effective for strain breeding. In this study, we first applied synchrotron irradiation to the filamentous fungus, *Monascus* sp. We successfully obtained two mutant strains SC02 and SC03 of *M. purpureus*, showing more than 2.5-fold MK production compared to the parental strain KUPM5, with high efficiency after screening approximately 900 colonies. Strain SC02 also produces secondary metabolites, *Monascus* pigments, comparable to strain KUPM5, whereas SC03 produced pigments at a lesser level, probably because of poor growth. Citrinin and *Monascus* pigments share the same precursor known as tetraketide, condensed by acetyl-CoA and malonyl-CoA. The production ratio of citrinin and pigments by *M. ruber* is regulated under environmental conditions, such as oxygen (141). Thus, as strain SC02 showed an increased production of both citrinin and pigments, the mutation of genes involved in the corresponding metabolic pathways to synthesize citrinin and the pigments probably altered the metabolic flow, resulting in the production ratio of final secondary metabolites.

Although we have not identified the genes responsible for the high level of production of MK even after comparative genome analysis, the results that strains SC02 and SC03 show higher production of both MK and citrinin than strain KUPM5 provide a clue to identify the genes. It is difficult to consider that the mutated genes gained their functions so that the two synthetic pathways of MK and citrinin are enhanced simultaneously. The mutated genes responsible for the hyperproduction of MK and citrinin are likely to be upstream regulators that commonly regulate

several synthetic pathways of secondary metabolites. LaeA is a putative histone methyltransferase, a master regulator of secondary metabolism in *Ascomycota*, including *Aspergillus* spp. and *Monascus* spp. (140). When the mutation occurred in the genes of regulator proteins, it might result in different types of changes in secondary metabolite synthesis and alterations in mycelium development and spore formation (142). Although mutations in the *laeA* ortholog have been found in the three mutant strains, both mutants SC02 and SC03 have increased the production of MK and citrinin. Therefore, it can be presumed that mutations in *laeA* have a favorable effect on the production of these secondary metabolites. To observe the effect of identified variant function, the type of genetic variant was predicted. All of the variations we discovered, showing missense mutation at the highest frequency rate about 90% but we found only one site of missense mutation occurred in an orthologs of *mokE* in SC03 mutant. The *mokE* gene was reported to provide an impact on the characteristics of mycelium, hyphae networking, sporulation and also MK synthesis in *Monascus*. Overexpression of *mokE* in *Monascus* M1 confirmed an enhancement of mycelium length consequently, stimulating the production of MK (143). It was hypothesized that mutation in *mokE* in SC03 strain is certainly influenced on the development of the morphological and in this instance, it had a smooth surface aspect that differentiates them from others and also overproduced MK than the parental KUPM5. Our study was then observed the variants in the set of *mok* gene orthologous and classified as a novel prediction. The majority of the variations found with MODIFIER impact, were putative regulatory variants altering gene expression, transcription factor binding and protein binding (144). These findings suggest the possibility that variant of MODIFIER impact corresponding to upstream, downstream, 5 prime UTR and 3 prime UTR regions have regulatory effects on the *mok* gene in different ways, which could modulate response to MK and others secondary metabolites production. Synchrotron light mutagenesis in *Monascus* showed a high SNP frequency and Indels of about 70% and 30%, respectively. Transversion substitution occurred at a high frequency of 92.38%, whereas transition substitution occurred at 7.62%. Our findings contradict with those from *S. cerevisiae* (129). The frequency of both transition and

transversion is reversed in *M. purpureus* and *S. cerevisiae*. In yeast, the transitional substitution pattern, in which the ratios of A:T to G:C and G:C to A:T were almost the same, was about 70%, and the transversion substitution patterns were about 30%.

Analyses of transition-transversion mutations in nature were performed in the *pks* gene in the various fungal strains of the *Aspergillus* section *Nigri* (145). During evolution of *Aspergillus* section *Nigri*, transition substitution occurred higher frequently than transversion did. The transition and transversion substitution rates induced by serial subculturing were also observed in the Ascomycete and Basidiomycete yeasts (146). The similar transition and transversion substitution rate were found in *S. cerevisiae.* the higher rate of transversion substitutions in *Schizosaccharomyces pombe,* and lower rate of those was found in *Rhodotorula toruloides*. The nucleotide substitution rates are depending on the species and are affected by concentration of cellular triglyceride and antioxidants, carotenoids in yeast (146). Thus, it is possible that high rate of transversion substitution in *Monascus* genome by synchrotron light irradiation is specific to *M. purpureus* but not due to synchrotron irradiation. The highest rate of A:T to C:G transversion substitution among transversion substitution types was found in the genome of all mutant strains of *M. purpureus* KUPM5. A:T to C:G transversion substitution is caused by defect in DNA repairing reactions. Mutations within the *mutT* and *pdc1* encoding 8-oxo-dGTP diphosphatase in *E. coli* and *S. cerevisiae* enhance A:T to C:G transversion substitution in the respective microorganisms (147, 148). Relative high rate of G:C to T:A transversion substitution was found in all *Monascus* mutant strains. The G:C to T:A transversion substitution occurs in defect in the mismatch repairing of DNA caused by oxidation reaction. The mutation within *mutY* encoding adenine glycosylase in *E. coli* cause a high rate of the C:G to A:T mutation (149). We identified the mutation site within *mutT* and *mutY* orthologous genes in three *Monascus* mutant strains. All mutant strains possess more than 4 nucleotide substitutions within the UTRs in the *mutT* and *mutY* orthologs (Table 4-1). The mutation sites are located 5'- and 3'-UTRs that may have an influence

on the transcription and stability of mRNA. Furtherer study will provide with evidences for high rate of transversion substitution compared to transition substitutions in the *Monascus* genome.

Though the different patterns of nucleotide substitution between *Saccharomyces* and *Monascus* in the same phylum *Ascomycota* are yet to be elucidated, synchrotron light irradiation is an alternative technique for fungal breeding. Thus, our discovery is groundbreaking in the field of synchrotron light-induced mutagenesis in *Monascus* mutants.

Chapter 5. Genome editing of *Monascus purpureus* using a CRISPR/Cas9 system

5.1. Introduction

Many metabolites and pharmaceuticals are produced by filamentous fungus. However, the severe contamination of fungal mycotoxins underlies a wide range of clinical disorders, from acute poisoning to long-term consequences such as immunological insufficiency and cancer, affecting several organ systems (150). Generally, *M. purpureus* produces beneficial secondary metabolites such as pigments, which are utilized as a food colorants (151) and monacolin K, hypocholesterolemic agent (35) . Meanwhile, also produce citrinin (75), a potent mycotoxin with nephrotoxic activity caused by the respiratory complex suppression (152). Citrinin was known as a major mycotoxin produced by *Monascus* spp. that contaminated in food ingredient in several countries. To prevent genotoxicity and carcinogenicity from citrinin-contaminated foods. The European Food Safety Authority has set the maximum citrinin level in food supplements based on rice fermented with red yeast *Monascus purpureus* at 100 µg/kg (31).

Traditional breeding of filamentous fungi using chemical and physical mutagens were effective, but it was time-consuming to select mutants and difficult to improvement due to a lack of information of the mutation sites, as well as difficult to isolate mutants as a homokaryon (153). The heterokaryotic mutants could still produce citrinin (82, 153, 154). Another effective approach for breeding of filamentous fungi is genetic engineering. Although this approach is a powerful tool for strain improvement but it still only used in the lab for basic research and no commercial applications have been recorded due to regulatory restrictions (155).

The clustered regularly interspaced short palindromic repeats (CRISPR) associated protein 9 (Cas9) system has enabled precise genome editing in many filamentous fungi (156). CRISPR/Cas9 is now the most widely used genome-editing technique. A Cas9 protein induces a double-strand break (DSB) in the target DNA consisting of a 20-bp sequence complementing the protospacer of the guide RNA (gRNA) with the 5'-NGG protospacer-adjacent motif (PAM) sequence. In recent years, CRISPR/Cas9 have been successfully altered in many fungal researches for instances; *Aspergillus* sp., *S. cerevisiae*, *T. reesei*, *N. crassa* and *Aspergillus* sp. (156), but *Monascus* research is still restricted. The functional alteration of the nucleotide sequence within biosynthetic gene cluster by gene disruption or genome editing can be applied to the filamentous fungi that had solved their genome information.

In this chapter, a mutant strain SC02 exhibit hyperproduction of monacolin K more than 3-folds was subjected to genome editing to reduce the citrinin production using the CRISPR/Cas9 genome editing system.

5-2. Materials and methods

5-2-1. Strains, plasmid, primers, and culture conditions

Escherichia coli DH5α was served as the general cloning host. The propagation of plasmid was grown in Luria-Bertani (LB) medium at 37℃ with 50 μg/ml ampicillin. *M. purpureus* SC02, MK hyperproducing strain derived from synchrotron light induced mutation in previous study (Chapter 4) were cultured on PDA medium at 30℃ for 5 days. ppAsATC9 plasmid was provided by Dr. Jun-Ichi Maruyama (Department of Biotechnology, The University of Tokyo, Japan) (157). Oligonucleotides were synthesized by Eurofins genomics Corporation (Tokyo, Japan), and used as primers this study, are listed in Table 5-1.

Table 5-1. Primers used in this study.

Oligonucleotides	Sequence (5' to 3')
citS-sgRNA	GCACTCAAATCTGAGATCAAAGGGTTTTAGAGCTAGAAATAGCAAGTTA AAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGC
F11-PU6	CTGCATATTGACGTAACAGGACCA
R11-PU6	CCTTTGATCTCAGATTTGAGTGCAATCCTAATAGTAAAATATATAGGAAG
F21-gRSF	CTTCCTATATATTTTACTATTAGGATTGCACTCAAATCTGAGATCAAAGG
R21-gRSF	CAAATATGCCTTCGGAAGGAAAGAGCACCGACTCGGTGCCACTTTT
F31-TU6	AAAAGTGGCACCGAGTCGGTGCTCTTTCCTTCCGAAGGCATATTTG
R31-TU6	TCTCCGCTACGAAGGCCAAAAAA
F1in-GEcitS1	TCGAGCTCGGTACCCGCATGCTCCCGGCCGCCATG
R1in-GEcitS1	AATTGCCCGATCCCCTCTCCCATATGGTCGACCTGCA
F1-citS2inv	GCACTCAAATCTGAGATCAAGTTTTAGAGCTAGAAATAGCAAGTT
R1-cit2inv	TTGATCTCAGATTTGAGTGCAATCCTAATAGTAAAATATATAGGAAG
FC-citS2	GCACTCAAATCTGAGATCAA
upcitS-F	CCACGAGATCAGCAAACGGT
upcitS-R	TCCCAGTGTTGGATTGCTGT

5-2-2. Protoplast preparation and transformation

M. purpureus SC02 was cultured on PDA plate at 30℃ for 5 days. Then, the spore suspensions were prepared and inoculated in PDB medium, incubated at 30℃, 160 rpm for 30 h. The mycelia were harvested and washed twice with 0.7 M NaCl. Subsequently, the mycelia were suspended in 10 ml of cell wall lytic enzyme solution containing 40 mg Yatalase Plus (Takara Bio, Japan) and 80 mg Cellulase (Yakult pharmaceutical, Japan) and then incubated at 30℃ for 2-3 h with shaking at 60 rpm, until protoplasts were formed. To remove cell debris from protoplasts, 10 ml of STC buffer (1.2 M sorbitol, 10 mM $CaCl_2$, 10 mM Tris-HCl pH 7.5) was added and filtrated. The filtrated protoplasts were centrifuged for at 3,500 rpm, 10°C for 10 min. The supernatant was discarded, and the pelleted protoplasts were washed with STC buffer at 4,500 rpm, 10°C for 5 min, then suspended in 1 ml of fresh STC buffer. For each transformation, 100 µl of protoplasts were prepared in 1.5 ml tube and placed on ice. Plasmid (5 µg) was added into protoplasts solution, mixed gently, and incubated on ice for 60 min. PEG4000 solution (1.25 ml 50% PEG4000, 50 mM $CaC1_2$, 50 mM Tris-HCI, pH 7.5) was carefully mixed, and incubated at room temperature for 20 min. The protoplasts were then spread onto SDA (4 g of soluble starch, 20 of g dextrose and 20 g of agar in 1 L of water) plate containing 0.1µg/ml of pyrithiamine and cultivated at 30℃ for 3 days until transformants were appeared.

5-2-3. Identification of *M. purpureus* U6 snRNA sequences

To find a homologous U6 RNA sequence in *M. purpureus* KUPM5, the U6 small nuclear (snRNA) of *A. oryzae* (Accession number MH032755.1) was used as a query, and a local BLASTN command was performed against the *M. purpureus* KUPM5 genome database. The identified sequences of U6 RNA genes with their 5'- and 3'- flanking regions covering 500 and 200 nucleotides, respectively, were used for multiple alignment analysis against *A. nidulans* (Accession number MH032752.1) and *A. niger* (Accession number MH032753.1) using Clustal Omega packaged with SnapGene software.

5-2-4. Construction of CRISPR/Cas9 plasmid

Plasmid ppAsATC9 harbors the expression cassette of Cas9, AMA1-based autonomously replicating sequences and the gene responsible for pyrithiamine resistance as a selection marker for transformation of *A. oryzae* (157). A ppAsATC9 contains a unique *Sma*I site where can be introduced sgRNA expression cassette. A sgRNA were designed to guide Cas9 to simultaneously cleave the specific site nearby upstream region target *citS* gene. PAM sequence should be present in the 3'end of the target DNA sequence. Thus, 20 nucleotides upstream of PAM sequence (5'-20 nt NGG-3') in *citS* were designed by the CRISPRdirect (158) and SnapGene software. To construct the genome editing vector ppAsATC9:citSko, the PU6 and TU6 (promoter and terminator) fragments were amplified by PCR from genomic DNA of *M. purpureus* KUPM5 using the sets of primers F1.1-PU6/R1.1-PU6 for promoter, and F3.1-TU6/ R3.1-TU6 for terminator. The gDNA including protospacer of *citS* gene target and sgRNA scaffold was amplified using the primers F2.1-gRSF/R2.1-gRSF and *citS*-sgRNA oligonucleotide as a template DNA. Three fragments were ligated to construct expression cassette PU6-gDNA-TU6 by overlap-extension PCR, using primers F1.1-PU6/R3.1-TU6. The expression cassette was ligated with linearized pGEM-T (Promega,

USA). PCR was done using the primer sets F1in-GEcitS1/R1in-GEcitS1, yielding the complete expression cassette of *citS*-targeting sgRNA driven by U6 promoter. The sgRNA expression was inserted into the *Sma*I site of ppAsATC9 using Infusion HD Cloning kit (Takara Bio, USA) to yield ppAsATC9:citSko (14.1 kb).

5-2-5. Screening of transformants showing low citrinin production

The *Monascus* transformants showed the resistance to pyrithiamine were screened using a plate bioassay based on the antibacterial property of citrinin, as described in Chapter 2. Briefly, *B. subtilis* NBRC13719 was cultivated on LB medium at 30°C for 24 hours. A 100 µl of the *Bacillus* culture was added to 8 ml of soft LB agar medium, and then was immediately poured onto a plate grown fungal transformants. The clear zone surrounding the fungal colonies was shown after 12 h. The fungal colonies showing narrow or absent of clear zones were selected.

5-2-6. Analyses of secondary metabolites

Dried red koji samples were prepared using the method described in Chapters 2 and 4. Briefly, 0.5 g of koji powder was extracted with 2 ml of 70% ethanol at 55°C for 1 h. The supernatants were then collected and filtered. Following that, the extracted samples were analyzed using HPLC on a C18 column (TSKgel ODS 4.6 mm 250 mm, Tosho, Japan). The condition for monacolin K was done using a mobile phase of 80% acetonitrile:0.1% phosphoric acid at a ratio of 60:40 and a UV detector at a wavelength of 238 nm. The mobile phase ratio for citrinin analysis was similarly 60:40, however a fluorescence detector with excitation at 330 nm and emission at 500 nm was used in case of citrinin. The RIDASCREEN FAST citrinin immuno-detection kit (R-Biopharm, Germany) were used according to the manufacturer's instruction to detect the citrinin.

Monascus pigment compounds were determined using a UV–visible spectrophotometer at 510, 480, and 400 nm to detect red, orange, and yellow pigments, respectively. One unit of the pigment yield was defined as showing 1 absorbance value at wavelengths of 510, 480, and 400 nm.

5-2-7. Analysis of *M. purpureus* mutants

To validate modifications at the targeted region, genomic DNA was extracted according to the procedure described in Chapter 3. PCR primers upcitS-F and upcitS-R were used to amplify the regions surrounding the target sequence. After purification, BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, USA) was used according to the manufacturer's instruction, and then sequenced by a DNA sequencer Spectrum Compact CE System (Promega, USA) to validate locus-specific alterations.

5-3. Results

5-3-1. Optimization of protoplast-mediated transformation

As far as we known, the CRISPR/Cas strategy has rarely been used in the genome editing of *Monascus* species. In filamentous fungi, transformation is required for introduction of a genome editing vector to the fungal cells. The efficiency of transformation is depending on the quality and quantity of the fungal protoplast. We initiated with the same method for protoplast formation as for *Monascus* spp. (159). There were only a limited number of resultant protoplasts were produced in the strain KUPM5 ($2.71x10^3$/ml). The procedure for protoplast formation suitable for strain KUPM5 was developed by varying the cultivation conditions and lytic enzyme incubation. The protoplast formation was optimized after cultivation of *Monascus* spores for 30 h and treated with lytic enzyme solution for 3 h to yield $3x10^6$/ml (Fig. 5-1A and B). The concentration of pyrithiamine used for selection of transformants is determined to be 0.1 μg/ml. Under the optimal condition for protoplast formation and selection in the presence of 0.1 μg/ml pyrithiamine, transformation of *M. purprueus* KUPM5 with plasmid pPTRII DNA (5 μg) harboring *ptrA* and AMA1 sequence gave a 19±3 colonies transformants. The improved procedures were used in subsequent protoplast transformations with Cas9 plasmid.

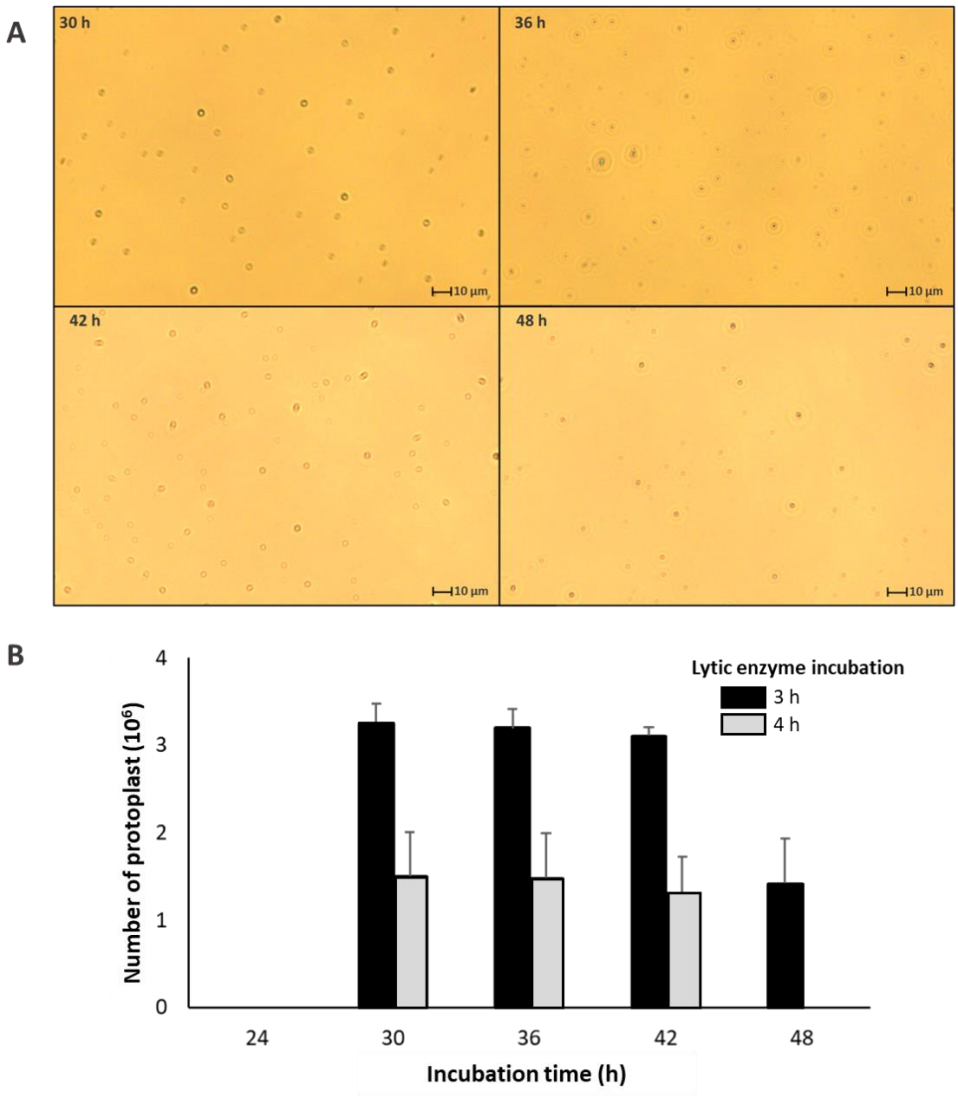

Fig. 5-1. Characteristics and number of protoplasts of *M. purpureus*. (A) An optical microscope picture of protoplasts acquired after optimizing the methods and (B) Number of protoplasts produced by varying cultivation time and lytic enzyme incubation time.

5-3-2. Identification of U6 sequences in *M. purpureus* by computational analysis

The U6 promoter and terminator from *M. purpureus* were chosen to express target gene sgRNA. Local BLASTN search using U6 RNA of *A. oryzae* as a query; homologous sequences with flanking regions of *M. purpureus* KUPM5 were identified in scaffold 1 with 99% identity to query sequences. Multiple sequence alignment revealed the characteristics of the *Monascus* U6 promoter consisting of consensus motifs (Fig. 5-2). With the nucleotide "G", the mature U6 RNA transcription start sites were initiated at position +1. The consensus motif TATA box (TATATA) was identified between positions -24 to -30 relative to the transcription start site (TSS) at upstream in the sequences. The putative U6 terminator containing B-box element was identified at the downstream of the TSS. The highly conserved nine residues (GGGTTCGAA) of B-box was localized at the position +181 to +190. Notably, it was homologous among the selected organisms *Monascus* and *Aspergillus*. As a result, these sequences were confirmed to be a putative U6 promoter and terminator of *M. purpureus* and then was utilized in the construction of the gRNA expression cassette.

Fig. 5-2. Multiple alignments of U6 RNAs with 5′- and 3′-fanking regions of *M. purpureus* against *Aspergillus* species. The consensus motifs of the upstream region and downstream region are shown. Transcription start site (TSS) was highlighted in red box.

5-3-3. CRISPR/Cas9 mediated mutagenesis of *citS* gene in *M. purpureus*

CRISPR Cas9 genome editing system requires two components: an endonuclease Cas9 and sgRNA. To study the viability and efficiency of the CRISPR/Cas9 system in *M. purpureus*, we targeted the *citS* gene, which encoded polyketide synthase, an enzyme in the citrinin production pathway (160). We hypothesized that the excision of *citS* gene may cause aberrant citrinin production. In this study, the plasmid ppAsATC9 was used as the backbone for the construction of the final expressed plasmid ppAsATC9:citSko. The ppAsACT9 plasmid comprises the AMA1 sequence for autonomous plasmid replication to enhance the efficiency of CRISPR/Cas9-mediated mutagenesis, and the pyrithiamine resistance marker was employed for screening (Fig. 5-3A). The *citS* gene target cut site was designed to be a 20-bp region of the protospacer sequence close upstream at the N-terminus (Fig. 5-3B). The U6 promoter of *M. purpureus* KUPM5 was chosen as a potent sgRNA transcriptional regulatory element for sgRNA transcription. The resulting construct plasmid ppAsATC9:citSko (Fig. 5-3C) was used for PEG-mediated protoplast transformation into *M. purpureus.*

In this study, a 20-bp *citS* protospacer, 5′-GCACTCAAATCTGAGATCAA-3′ with protospacer adjacent motif (PAM) AGG-3’ was designed for inactivation of *citS* gene *in M. purpureus* SC02. This region is located nearby first start codon of the gene. The expression plasmid ppAsATC9:citSko was introduced into *Monascus* protoplasts through PEG-mediated transformation. The protoplasts were cultured for 3 days until colonies appear. A total of 148 transformants were appeared and two colonies (termed CR1 and CR2) displayed the narrow clear zone that are smaller than wt did (Fig. 5-4). To characterize CRISPR/Cas9-induced alterations, genomic DNA of the mutants were amplified around target site using upcitS-F/upcitS-R primers. PCR produced 354 bp amplicons which were subsequently sequenced. DNA sequencing results revealed that only mutant strain CR2 had a single T-base deletion near the PAM region (Fig. 5-5), while no base substitution occurred in a particular location of strain CR1.

5-3-4. Citrinin production from *Monascus* mutants

To investigate the remaining citrinin production, mutant and wt strains were cultured on steamed rice to make red koji. The samples were collected at 7th day of cultivation and then analyzed by HPLC. The retention times of citrinin were same at 12.5 min (Fig. 5-6A). Only mutant CR2 which had 1 base deletion, retained 48% of citrinin production (18.71±4.56 ng/g) whereas mutant CR1 produced citrinin same to wt made (36.22±4.23 ng/g) (Fig. 5-6B).

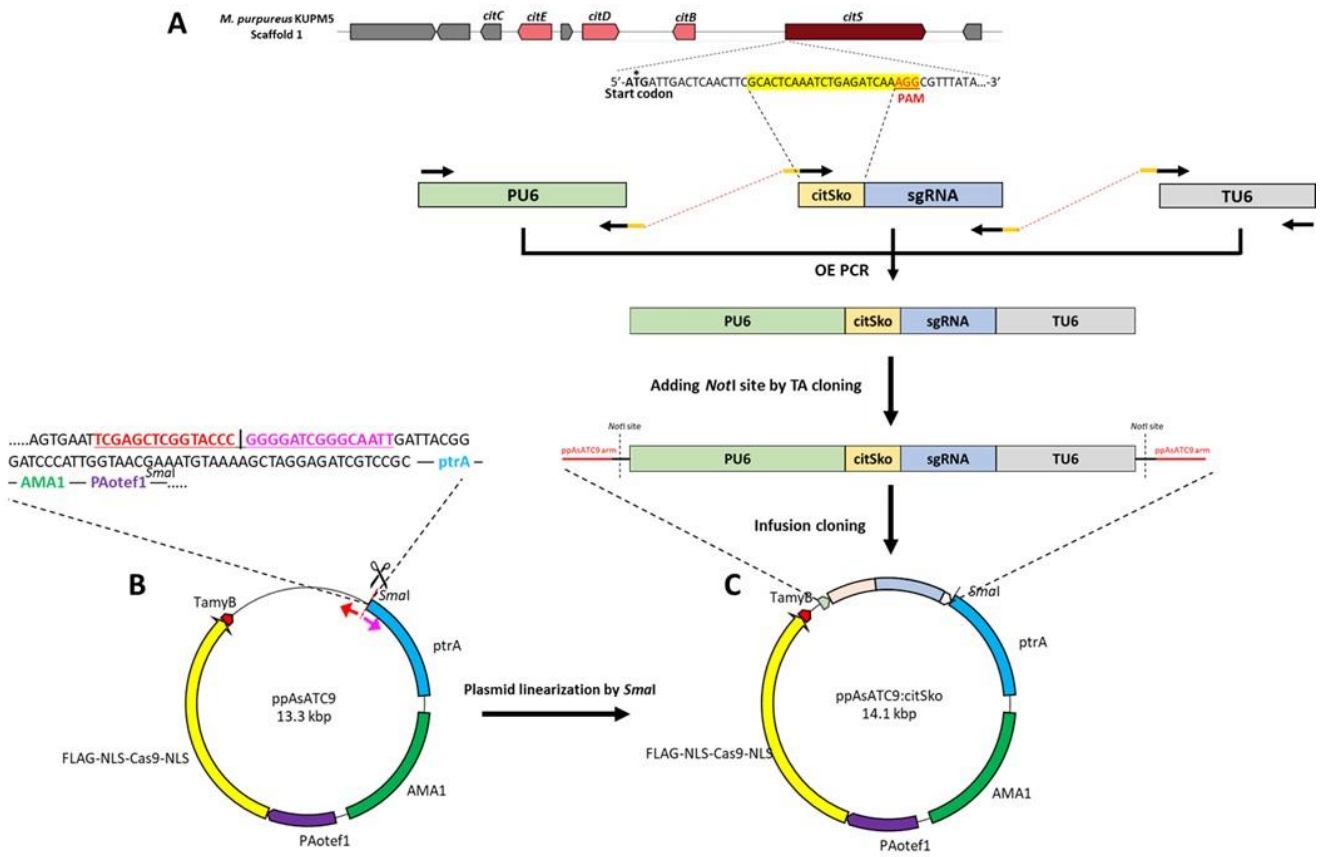

Fig. 5-3. Construction of ppAsATC9:citSko plasmid for genome editing. (A) The fragment U6 promoter and terminator, and protospacer with the sgRNA sequence were connected by overlap-extension PCR, yielding PU6-citSko-sgRNA-TU6. (B) The PU6-citSko-sgRNA-TU6 was cloned into pGEM-T easy vector to add *Not*I site and (C) The completed expression cassette was fused with backbone plasmid ppAsATC9 by infusion cloning, producing ppAsATC9:citSko.

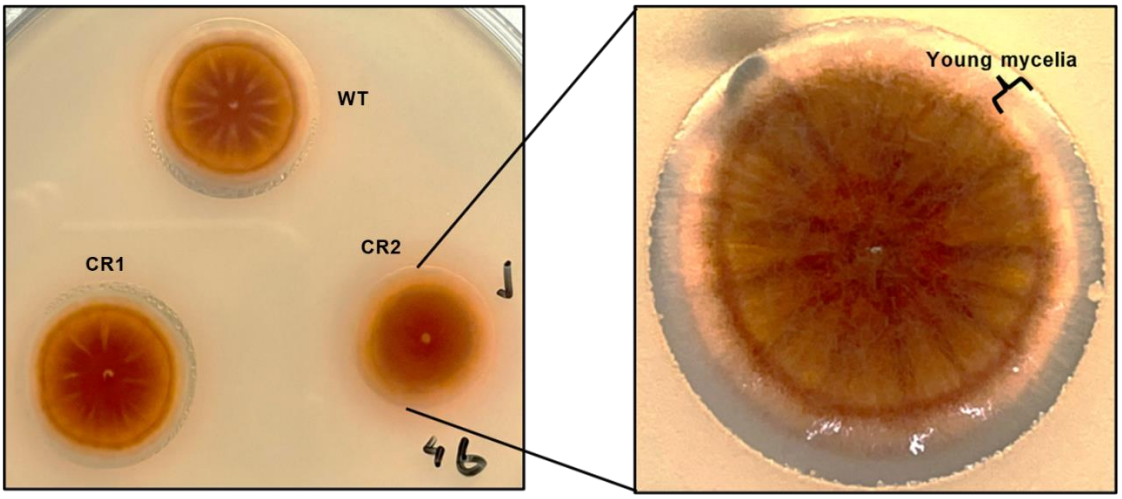

Fig. 5-4. The inhibitory zone of *Monascus* mutant strains after a *B. subtilis* plate bioassay. *Bacillus* culture (in soft LB agar medium, 0.7% agar) was placed onto the plate for screening after all mutant strains had been grown at 30°C for 3 days. The image on the right showed a close-up of mutant CR2, which seemed to have a tiny clean zone.

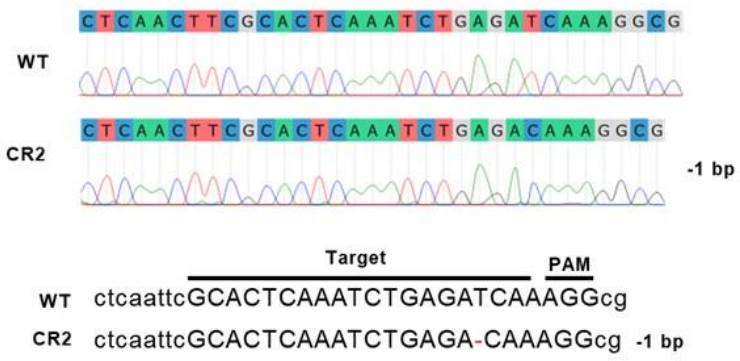

Fig. 5-5. Targeted mutagenesis of *citS* gene using CRISPR/Cas9 system. Sanger sequencing of the wt and mutant strain CR2. The locations of the mutations were shown in red hyphen.

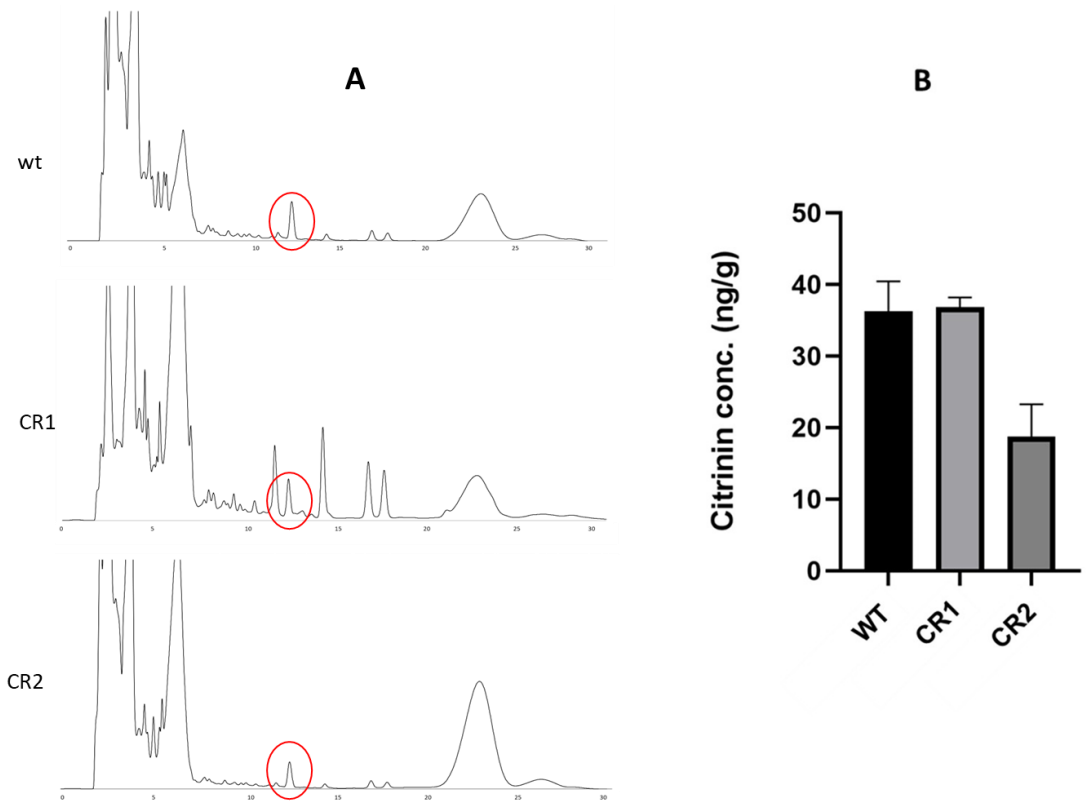

Fig. 5-6. Citrinin production of *M. purpureus* strains. (A) HPLC chromatogram of citrinin in koji extracts from wt (SC02) and mutants. The retention time of citrinin was detected at 12.5 min. (B) Citrinin concentration derived by wt and mutants.

5-3-5. Pigments and monacolin K production by selected mutant

Monascus also produces secondary metabolites, including azaphilone pigments (red, orange, and yellow) and monacolin K. After 7 days of cultivation, the pigments of *Monascus* koji were measured (Fig. 5-7A). Mutant strain CR2 produced red, orange, and yellow pigments with yields of 16.95±2.05 U/g koji, 15.65±2.23 U/g koji, and 19.14±2.17 U/g koji, respectively, that did not show significant differences from those of wt. In term of monacolin K, the yield of CR2 was 5.86±2.02 mg/g koji, which was comparable to what wt produced at 5.76±1.44 mg/g koji (Fig. 5-7B).

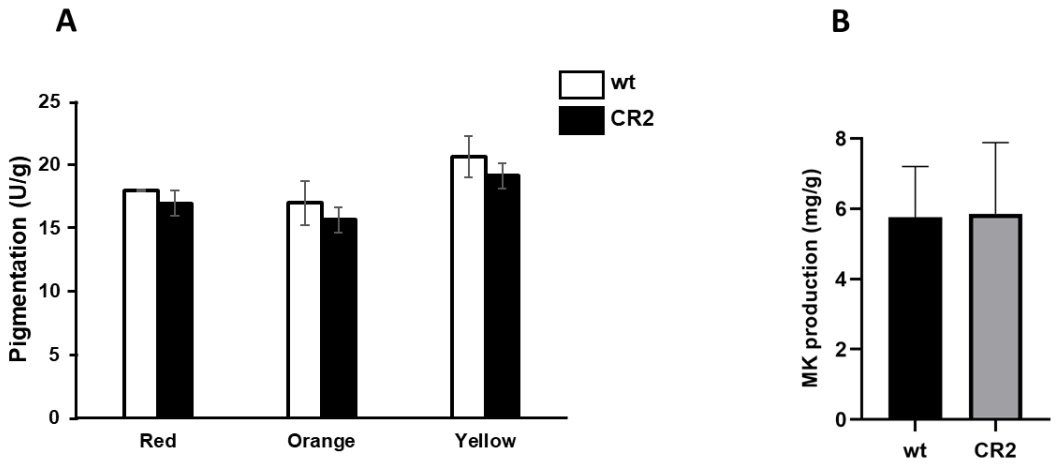

Fig. 5-7. Secondary metabolite production in red koji made by mutant CR2 compared to wt strain NS02 after 7 days of cultivation. (A) *Monascus* red, yellow, and orange pigments and (B) Monacolin K production. All experiments were conducted in triplicate.

5-4. Discussion

Citrinin is a kind of mycotoxin produced via the polyketide pathway by filamentous fungi, mainly by *Monascus* spp. and *Penicillium* spp. (34). Containing full length of PKS gene (*citS*) and six additional genes encoding putative dehydrogenase, $Zn(II)_2Cys_6$ transcriptional activator, oxygenase, oxidoreductase, and membrane transporter (7). According to several researches, disrupting *pksCT* (known as *citS*) might abolish or limit citrinin synthesis (153, 160-162). Before initiating gene editing, it is also essential to determine the proper plasmid composition. Promoter type influences the efficiency of expression of cas9 protein and sgRNA in CRISPR/Cas9 system (163). Although it has been reported that RNA polymerase II promoters such as *gpdA* is strong constitutive promoter for driving the expression of sgRNA in *M. purpureus* especially, in CRISPR/Cas9 approach (164). U6 promoters, driven by RNA pol III were also effective in this approach because of their ability to prevent post-transcriptional modifications (165). However, it has not yet been characterized in *M. purpureus*. Here, we first identified the RNA pol III promoter in *M. purpureus* strain KUPM5.

Pyrithiamine is toxic to yeast cells and also filamentous fungi such *Aspergillus* by disrupting thiamine pyrophosphate, a required co-factor for cell enzymes (166). Pyrithiamine can be utilized in *Monascus* at the concentration of 0.1 µl/ml. This is the first report on the transformation utilizing pyrithiamine in *Monascus*, and it sets the foundation for future research.

In this study, we attempted to introduce CRSPR/Cas9 system targeting the *citS* gene that is responsible for the production of citrinin, into *M. purpureus* monacolin K hyperproducing strain SC02. This allows the mutants to inactivate the function of *citS*, which in turns loss of production of citrinin. The specific target for Cas9 protein cleavage through this approach was located in the upstream region near start codon of *citS* gene at position between +18 to +37. The results showed, *citS* gene of the strain CR02 was knockout by 1-base deletion three base upstream the PAM

sequence, which is general cleavage site of Cas9. Our study demonstrated that ppAsACT9:citSko could be functional in *Monascus*.

The efficiency of isolation of genome edited strain was low, a single strain CR02 is obtained from 148 transformants. The carbon source in a selective medium, such as starch, dextrin, or maltose, is one of the most influential factors in driving Cas9 expression via the *A. oryzae* amyB promoter. In this study, SDA medium without source of nitrogen supplemented with pyrithiamine was used for transformants screening. The absence of nitrogen in the selective SDA medium may cause the suppression of *cas9* expression (157, 167, 168) which in turn resulted in reduced activity of cleavage of target site catalyzed by Cas9. The sole genome edited strain CR02 does not eliminate citrinin production but still retained about 48% as judged by the HPLC analysis. Shimizu *et al*. reported that *citS*-disruptant still produced the peak corresponding to citrinin on the HPLC analysis, but citrinin is not detected by the immuno-detection kit for citrinin in the same sample (32). However, subsequent testing process with immuno-detection kit, it was found that strain CR2 was capable of detecting citrinin. In this experiment, strain CR2 showed its low capacity of producing citrinin. It might be that gene knockout of *citS* will not terminate the production of citrinin and there might be presence of genes than *citS* may be involved in citrinin biosynthesis in *M. purpureus* (161). Other polyketides from the *pks3* gene are reported to be responsible for catalyzing multiple steps in the pigment biosynthesis pathway in ascomycetes, identified in *Monascus* species as the MpPKS5 gene. In addition to monascorubrin biosynthesis, the *pks3* gene corresponds to the production of the well-known toxin citrinin as well as ankaflavin, a yellow pigment (169, 170). It cannot exclude the possibility that the strain CR2 is a heterokaryon carrying mutant *citS* and wt *citS* gene. Furthermore, in *M. aurantiacus* and *M. purpureus*, deletion of the gene *citS* resulted in a dramatic decrease in citrinin production, but not its elimination (161, 171). The amounts of azaphilone pigments and monacolin K derived by strain CR2 were comparable to wt produced. As a result, this CRISPR/Cas9 method is indeed high sequence-specific and does not interfere with or cause errors elsewhere on the gene.

In summary, we have demonstrated that CRISPR/Cas9 can be developed as a tool for gene disruption in *M. purpureus*. The further development of this technology is underway and holds significant potential for advancing the state of genetic research in this organism and, consequently, allow to construct superior *Monascus* strain with high level of functionality and safety.

Chapter 6. Conclusion

In recent years, secondary metabolites derived by *Monascus* spp. have attracted worldwide attention and been used in industry due to their beneficial effects. *Monascus* spp. have been shown to produce a variety of compounds, including *Monascus* pigments, monacolin K, and citrinin. Although, *Monascus* spp. produce advantageous secondary metabolites, some strains may produce a mycotoxin. Thus, the fermented food produced by *Monascus* species has proven to be problematic and contentious.

Therefore, we concerned about the long-term consequences of consuming citrinin-contaminated food. This dissertation has focused on the development of *M. purpureus* strains that produce more beneficial MK than the wild-type strain KUPM5 while simultaneously eliminating the synthesis of the harmful citrinin. First, we induced mutations in *M. purpureus* KUPM5 using traditional mutagenesis techniques, such as UV irradiation and NTG chemical treatment, in order to isolated mutant strains with low citrinin production while retaining enzyme activities for supply to the food industry. Consequently, we identified the mutant strains KS301U and KS302U that produced 80% less citrinin and retained the ability to produce red pigments in red koji compared to the wild-type KUPM5. The activity of α-amylase, protease, and lipase in red koji extract made by strain KS302U was greater than that of mutant strain KS301U, which was comparable to that of wild-type KUPM5.

Traditional mutagenesis using chemical and physical mutagens has shown to be an excellent method for improving microorganisms, as demonstrated by the findings of prior research. Subsequently, we performed mutagenesis using a different technique, resulting in an increase in beneficial secondary metabolites known as monacolin K in koji. Therefore, we developed mutant strains with an increased ability to produce MK after irradiation of synchrotron light. We successfully isolated the mutant strains SC02 to produce MK threefold higher than strain KUPM5 while retaining the production capabilities of other metabolites, such *Monascus* pigments, mycelial

contents, and α-amylase activity comparable to strain KUPM5. The mutation pattern caused by synchrotron light irradiation was elucidated by comparative genome analysis. As a result, the mutation rate of around 90% of all genes was induced by synchrotron light, with 92.38% of transversions and 7.62% of transition base alterations. Our findings demonstrated that synchrotron light irradiation was extremely effective for the strain improvement of a filamentous fungus, *M. purpureus*, and provided information on the mutational features of this mutagen.

Simultaneously, a substantial breakthrough in our knowledge of molecular information was not yet widely publicized, and many areas lacked clarity. The whole genome analysis of *M. purpureus* strain KUPM5 revealed that genome size is 24.47 Mb and codes 9,270 predicted genes. In addition, the MK biosynthetic gene clusters (BGCs) are presented in three separated loci. Citrinin and *Monascus* pigment biosynthetic gene clusters in the genome of KUPM5 were preserved with those of other *Monascus* spp. The identification of BGCs has unquestionably increased our understanding of the biosynthetic pathways of secondary metabolites in *Monascus*, providing a theoretical foundation for commercial production of *Monascus* secondary metabolites.

Genome editing facilitates the alteration of a desired gene by deleting, inserting, or replacing DNA in a sequence-specific ways. Thus, we introduced the CRISPR/Cas9 system into *M. purpureus* SC02 in order to specifically inactivate the *citS*, the crucial gene in the citrinin biosynthetic gene cluster. A mutant strain with 1-base deletion with the *citS* gene was isolated indicating that our CRISPR/Cas9 system constructed in this study are functional in *M. purpureus* KUPM5. The optimized expression of Cas9 and sgRNA in *Monascus* strains allow to efficient generation and isolation of the genome edited clones.

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