Analysis of the inhibitory effect of rapamycin on *Fusarium asiaticum* by next generation RNA sequencing

Yumiko Iwahashi

1Food Research Institute, National Agriculture and Food Research Organization, 2-1-12 Kannondai, Tsukuba 305-8642, Japan

**Abstract**

*Fusarium asiaticum* was used to investigate factors affecting the regulation of deoxynivalenol (DON) production. In liquid medium supplemented with a low concentration of rapamycin, *F. asiaticum* grew at approximately 40% of its normal rate but did not produce DON. RNA levels were measured by next generation RNA sequencing. In low concentration rapamycin, expression levels of DON-related genes were low, but expression of the *FaTor* (target of rapamycin of *F.asiaticum*) gene was not suppressed. However, expression of the *FaTap42* gene, which interacts with *FaTor*, was suppressed to nearly 70%. *FaSit4* and *FaPpg1* genes, which were downstream of *FaTap42*, were down regulated. However, expression of the *FaPp2A* gene, another phosphatase downstream of *FaTap42*, was not affected by low concentration rapamycin. These findings reveal that low concentration rapamycin differentially suppresses genes downstream of the *FaTor* pathway.

**Introduction**

Red molds are phytopathogenic fungi that cause yield reduction of agricultural products worldwide through the production of toxic mycotoxins [1]. The *Fusarium graminearum* species complex consists of at least 13 phylogenetically different species [2]. These species tend to produce different strain-specific trichothecenes, including nivalenol, deoxynivalenol (DON), 3-acetyldeneivalenol and 15-acetyldeneivalenol [3]. Geographically, among the members of the *F.graminearum* species complex, *F. asiaticum* is more common in temperate Asian regions where average annual temperature is lower than tropics regions [4]. *F. asiaticum* produces various mycotoxins, including DON.

DON binds to ribosomal peptidyltransferase and affects protein synthesis [5-7]; therefore, it is possible that long-term intake of small quantities of DON can cause immunodeficiency [8]. Furthermore, DON induces inflammation of intestinal epithelial cells through the release of reactive oxygen species [9]. A large - scale survey of urine from children in the UK has shown that even a normal diet risks exposure to DON [10]. The long-term effect of DON intake over several decades is unknown; however, DON exposure throughout life is likely to be harmful.

The trichothecene biosynthetic pathway in *fusarium* is largely determined [11], but the regulatory mechanisms of the genes involved are not fully elucidated. Most DON-related genes reside in clusters containing two transcriptional regulators (Tri6 and Tri10) [2]. In recent years it has become clear that regulation of secondary metabolite biosynthesis in most fungal genera is responsive to the external environment and that various regulatory systems are involved in its regulation [13].

The levels of many metabolites are increased in DON producing *F. asiaticum* cells [14]. Glycolytic metabolite levels are markedly increased, whereas pyruvic acid is reduced and pyruvate dehydrogenase kinase is increased [15]. Pyruvate dehydrogenase supplies pyruvic acid generated in the glycolysis system to the TCA cycle. However, in producing DON, pyruvate dehydrogenase kinase is activated to suppress pyruvate dehydrogenase, which prevents self-destruction of mitochondria, and ensures the production of factors necessary for cell proliferation [16]. These phenomena are similar to the Warburg effect commonly found in cancer cell metabolism [15].

It is known that the Warburg effect of certain cancer cells involves the change of Tor (target of rapamycin) kinase pathway [16]. The Tor kinase was first discovered in the budding yeast *Saccharomyces cerevisiae* [17] and it plays an important role in various eukaryotic signaling pathways [18]. Rapamycin, a macro-

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**Correspondence**

Yumiko Iwahashi, Chemical Hazard Unit, Food Research Institute, National Agriculture and Food Research Organization, 2-1-12 Kannondai, Tsukuba 305-8642, Japan.

E-mail: yumiko@affrc.go.jp

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lide antibiotic, led to the discovery of this Tor kinase pathway. Rapamycin was discovered as an antifungal agent against the pathogenic bacteria, *Candida albicans* [19]. Rapamycin was later discovered to inhibit proliferation of mammalian cells and to have immunosuppressive properties. The Tor kinase pathway inhibited by rapamycin plays an important role in eukaryotic nutrient and cell cycles [20, 21].

The Tor kinase of *S. cerevisiae* or mammalian forms two complexes known as TORC1 and TORC2 [22]. TORC1 activates assimilation processes, such as development, protein synthesis, transcription and nutrient uptake [22]. In addition, by inhibiting catabolic processes such as autophagy and ubiquitin-dependent proteolysis, it mediates transient regulation of cell growth [22]. TORC2 is insensitive to rapamycin and is involved in the organization of the actin cytoskeleton, endocytosis, and genome stability [23, 24]. Tor kinase is also related to aging, and dietary rapamycin increases the life span of mice [25].

*F. asiaticum* producing DON behaves similarly to some actively proliferating cancer cells [14]. Here, we examined how rapamycin influences the regulation of DON production in *F. asiaticum* using RNA sequencing. Next-generation sequencing is rapidly becoming the method of choice for transcription profiling experiments. It is possible to detect lower abundance transcripts compared with microarray technology and to identify new transcripts. Furthermore, it does not require a sequenced genome. In addition, unlike hybridization-based detection, RNA-seq allows analysis of genome-wide transcription at the single nucleotide resolution, enabling identification of alternate splicing events and post-transcriptional RNA editing events [26].

A low concentration of rapamycin inhibited the production of DON while not inhibiting *F. asiaticum* growth partially. The level of FaTor RNA was not affected by rapamycin treatment; however, the RNA level of FaTap42, which is downstream of FaTor and a common regulatory subunit for all three PP2A family members [27], was suppressed to about 70% by a low concentration of rapamycin treatment. In this paper, the relationship between suppression of DON production and FaTor pathway for low concentrations of rapamycin treatment in *F. asiaticum* is discussed.

**Stocks, media, growth conditions and chemicals**

*Fusarium asiaticum* strain MAFF 111889 was used in this study. This strain was isolated from maize in a field (Tochigi, Japan), and was identified and deposited in Gene bank project, NARO. *F.asiaticum* MAFF 111889 produces DON when 2 mM agmatine is added to minimal medium, but does not produce nivalenol in any medium. The strain was maintained on potato dextrose agar (Japan Becton-Dickinson, Tokyo, Japan). For the quantification of DON and to measure the growth curve, a modified Czapek liquid medium (pH 7.0) containing 30.0 g/l sucrose, 2.0 g/l NaNO₃, 15 mM MgSO₄ and 2 mM agmatine (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) was used. Conidia or mycelia of *F. asiaticum* were inoculated into potato dextrose liquid medium and preincubated at 25°C with shaking at 100 rpm for 3 days. Conidia or mycelia of preincubated samples were collected by filtration and 100 mg were inoculated into medium containing 2 mM agmatine or 2 mM agmatine + 5×10⁻⁸ mg/ml rapamycin and incubated at 25°C with shaking at 100 rpm for 5 days. 18 cultures of each with 2 mM agmatine or 2 mM agmatine + 5×10⁻⁸ mg/ml rapamycin were performed, the total wet weight and DON concentration were measured daily, the average of three cultures was determined. Growth curves were expressed as log of wet weight of conidia or mycelia after filtration.

**Determination of DON yield**

DON concentration in the culture filtrate was determined using a DON kit (Frontier Institute, Hokkaido, Japan) according to the manufacturer’s instructions. Culture filtrates were initially diluted in phosphate-buffered saline (pH 7.5) to provide a detection range of 0.5–20 ppm.

**Rapamycin or thiophanate-methyl treatment**

To test the effects of rapamycin (Adipogen, Liestal, Switzerland) or thiophanate-methyl (Sumitomo chemical, Tokyo, Japan) on *F. asiaticum* growth, an 8 mm diameter paper disc (Advantec, Tokyo, Japan) was placed on potato dextrose agar, and rapamycin or thiophanate-methyl was added directly thereon, followed by inoculation with conidia or mycelia (10 mg put directly on to the disc). Plates were cultured at 25°C for 3 days in the dark. The inhibition rate was calculated from the average colony area of 3 plates.

**RNA Preparation**

Conidia or mycelia grown in liquid culture were filtered and frozen under liquid nitrogen. Total RNA was crude extracted from 1 g of frozen sample with RNAiso Plus and then purified using Nucleo Spin RNA Clean-up EX, according to the manufacturer’s instructions (TAKARA Bio, Shiga, Japan).

An Agilent 2200 TapeStation (Agilent Technologies, California, USA) was used to assess the quality and to quantify nucleic acids. PolyA⁺ RNA was isolated, fragmented and used to synthesize single-stranded cDNA by reverse transcription. Double-stranded cDNA incorporating dUTP was then synthesized from this template. After polishing and phosphorylating both ends of the obtained double-stranded cDNA, a 3’-dA protruding treatment was performed and the index adapter was ligated. A sequence library was prepared by performing PCR amplification using a double-
stranded DNA template to which an adapter was ligated and a polymerase that does not selectively amplify a strand containing dUTP. For preparation, 4 μg of cDNA was used as a template, and 15 cycles of PCR were performed. Sequence library quality was assessed using an Agilent 2100 BioAnalyzer.

**Sequence analysis**

The number of bases read for sequence analysis was 100, and the paired end method was used. Table 1 shows the equipment and manual name used for cluster formation and sequence analysis.

**Mapping and annotation**

Data mapping and annotation were performed using Genedata Profiler Genome software (Version 10.1.15a, Genedata Offices, Basel, Switzerland). Data were mapped to the lead sequence obtained by sequence analysis using STAR (2.5.2b). Annotation was then performed from genome position information obtained by the mapping. The annotation referred to the genomic and gene sequences of *Fusarium graminearum* PH-1 (http://www.ncbi.nlm.nih.gov.genome/ 58?genome=assembly_id=284609). The genomic sequence and genetic information referred to were obtained on October 12, 2018 from URL (ftp://ftp.ncbi.nlm.nih.gov/genomrd/all/GCF/000/240/135/GCF_000240135.3_ASM24013v3/).

**Mapping results**

Statistical values obtained by mapping the lead sequence to the genome are shown in Table 2. The products extracted from three independent cultures were measured separately. The quantitative value expresses the average of each measured value.

**Normalization processing**

Long sequence lengths are more frequently produced than short lengths. Therefore, gene length and so on mapping lead number corrected the number of mapped fragments, and expression level was expressed as FPKM (Fragment Per Kilobase of exon per Million mapped fragments).

**Results**

**Rapamycin strongly inhibits the growth of *F. asiaticum***

The inhibitory effect of rapamycin on *F. asiaticum* growth was unknown; therefore, the effect of rapamycin was assayed. Thiophanate-methyl, used as a fungicide for red mold fungi, was used as a control for growth inhibition. Since no reference was found that was directly examined by placing rapamycin or thiophanate-methyl on the disc, experiments were

| Table 1 | Equipment, reagents, and manual names used for cluster formation and sequence analysis. All are from Illumina K.K. (Tokyo, Japan) |
|---------|----------------------------------------------------------------------------------------------------------------------------------|
| Cluster formation | Equipment name | cBot |
| Reagent | HiSeq PE Rapid Cluster Kit v2-HS |
| Manual | cBot User Guide Rev.L |

| Sequence analysis | Equipment name | HiSeq 2500 |
| Reagent | HiSeq Rapid SBS Kit v2-HS |
| Software | HiSeq Control Software (HCS) v2.2.68 |
| Manual | HiSeq 2500 User Guide Rev.B |

| Table 2 | Mapping lead sequences to the genome sequence. |
|---------|------------------------------------------------|
| Agmatine | Agmatine+Rapamycin |
| No 1 | No 2 | No 3 | No 1 | No 2 | No 3 |
| Total Read [Count] | 52158330 | 51235352 | 52347897 | 52256870 | 51987845 | 52890976 |
| Mapped Read [Count] | 45437116 | 46137313 | 46524387 | 50129034 | 48976489 | 50198726 |
| Mapped Read [%] | 87.1 | 90 | 88.8 | 95.2 | 94.2 | 93.9 |
conducted by serially diluting from 1 mM/ml concentration in order to examine the inhibitory effect of both. Growth of *F. asiaticum* was completely inhibited by 1.25 ng of rapamycin on the disc placed in the PDA medium (Fig. 1-A). Thiophanate-methyl showed only about 10% growth inhibition at 4.5 µg (Fig. 1-A). In *F. asiaticum*, rapamycin inhibits growth and this inhibitory effect more than $1 \times 10^4$ times greater than that of thiophanate-methyl, which is a component of many red mold control agents.

**Inhibition of DON production by rapamycin**

As *F. asiaticum* used in this experiment does not produce DON unless agmatine, *F. asiaticum* was incubated in Czapek medium with 2 mM agmatine at 25°C for 5 days. When rapamycin at $5 \times 10^{-8}$ mg/ml was added to the above medium, *F. asiaticum* grew to about 40% of the control (the wet weight of cells cultured for 5 days was compared). Furthermore, DON production was completely suppressed in rapamycin-supplemented medium (Fig. 1-B, C).

**Next generation RNA sequencing in *F. asiaticum***

Among the 13,313 *F. asiaticum* genes that could be detected by RNA sequencing, 2% increased their expression by 5-fold or more in rapamycin-treated cultures. Similarly, 23% of genes were suppressed by rapamycin to 0.5-fold or less of the baseline levels. Furthermore, 48% of genes suppressed to 0.5 or less by rapamycin treatment were related to RNA degradation or the ribosome biogenesis in eukaryotes/RNA transport/mRNA surveillance pathway (data not shown).

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*Fig. 1* Inhibition of *F. asiaticum* growth by rapamycin and suppression of DON production.

A: A paper disc was placed in the center of the PDA plate, 4.5 µg of thiophanate-methyl or 1.25 ng of rapamycin was then placed on the disc and the plate was inoculated with 10 mg/wet weight of conidia or mycelia on disk. Plates were incubated at 25°C for 3 days in the dark. The inhibition rate was calculated from the average colony area of 3 plates.

B: Conidia or mycelia of preincubated samples were collected by filtration and 100 mg were inoculated into medium containing 2 mM agmatine or 2 mM agmatine + $5 \times 10^{-8}$ mg/ml rapamycin and incubated at 25°C with shaking at 100 rpm for 5 days. 18 cultures of each with 2 mM agmatine or 2 mM agmatine + $5 \times 10^{-8}$ mg/ml rapamycin were performed, the total wet weight is measured daily, the average of three cultures was determined. Growth curves were expressed as log of wet weight of conidia or mycelia.

C: The amount of DON produced was measured by filtering the culture solution and diluting to the measuring range.
Suppression of DON-related genes by rapamycin

RNA levels in conidia or mycelia cultured with $5 \times 10^{-8}$ mg/ml rapamycin were examined by next generation RNA sequencing. As shown in Table 3, the expression of DON-related genes was scarcely suppressed.

Changes in FaTor-related gene expression in response to rapamycin

The concentration of rapamycin that did not completely inhibit growth did not inhibit the expression of FaFkbp12 (FK 506 binding protein 12 kDa) and FaTor genes (Table 4). However, the expression level of the FaTap42 gene, which is a downstream component of the FaTor complex, was reduced to about 70%. Furthermore, the expression was decreased for FaSit4 and FaPpg1 genes, phosphatases that interact with FaTap42 (Table 4). In addition, expression of FaMgv1 and FaMsg5 genes downstream of FaSit4 and FaPpg1 were suppressed. Similarly, expression of the FaAreA gene downstream of FaPpg1 was also suppressed (Table 4). On the other hand, the expression of the FaPp2A gene downstream of FaTap42, which belongs to the FaTor pathway, was not suppressed by low concentrations of rapamycin (Table 4).

Discussion

RNA-seq is a sequencing analysis method that chooses mature mRNAs with poly-A tails. These mRNAs are then fragmented, converted to cDNA, and then a sequence adapter is added to both ends. Reading the expression of RNA under certain conditions with a next generation sequencer and counting the number of sequences generates the expression profile. In this study, the number of F. asiaticum mRNAs that could be detected with RNA-seq increased by 11% compared with our microarray studies. For comprehensive analysis of mRNA, RNA-seq will become the method of choice.

We have used DNA microarrays and LC-MS/MS to study the regulation of DON production. In the previous paper, when producing DON, the method of energy acquisition differs from when not producing DON in F. asiaticum. Metabolites of the glycolytic system and pentose phosphate pathway are increased, which is similar to the Warburg effect of cancer cells. Furthermore, when producing DON, genes related to Tor pathway were activated in F. asiaticum (data not shown). Therefore, the inhibitory effect of rapamycin in F. asiaticum was experimented. The control sample was added agmatine because F. asiaticum strain could not produce DON without agmatine.

Although there is a little available information on the Tor pathway of phytopathogenic fungi, rapamycin acts against Cryptococcus neoformans, Aspergillus species and Phodospora anserine and against the phytopathogenic fungus, Botrytis cinerea. In this study, rapamycin also strongly inhibits the growth of F. asiaticum (Fig. 1-A). In genome-wide searches for Tor kinases, only a single Tor ortholog has been identified in Aspergillus species, Fusarium graminearum, Neurospora crassa and Magnaporthe oryzae.

In S. cerevisiae, rapamycin forms a complex with the peptidyl-prolyl cis/trans isomerase, Fkbp12, which then binds to Tor kinase and inhibits kinase activity.

Table 3 Tri genes cluster expression after rapamycin treatment. RNA was extracted from cells cultured at 25°C for 5 days in a liquid medium supplemented with 2 mM agmatine or 2 mM agmatine + 5 mg$10^{-8}$ mg/ml rapamycin and the RNA expression level was measured using the RNA sequencing method. As there is no genome database of F. asiaticum, gene functions of the Tri gene cluster based on the genome sequence NC_026475.1: Fusarium graminearum PH-1 isolate (assembly ASM24013v3) and on GenBank annotations and annotations reported by Proctor et al.
Table 4  FgTor-related gene expression after rapamycin treatment. RNA was extracted from cells cultured at 25 °C for 5 days in a liquid medium supplemented with 2 mM agmatine or 2 mM agmatine + 5×10^{-8} mg/ml rapamycin and the gene expression level was measured using the RNA sequencing method. As there is no genome database of *F. asiaticum*, gene functions of the FgTor pathway based on the genome sequence NC_026475.1: *Fusarium graminearum* PH-1 isolate (assembly ASM24013v3) and on GenBank annotations and annotations reported by Proctor et al.\textsuperscript{43}

| Locus Tag     | agmatine+ rapamycin/ agmatine (Fold change) | Description                                      |
|---------------|---------------------------------------------|--------------------------------------------------|
| FGSG_09690    | 1.73                                        | FaFKbp12 (FK506 binding protein)                 |
| FGSG_08133    | 1.76                                        | FaTor (target of rapamycin)                      |
| FGSG_09800    | 0.72                                        | FaTap42 (serine/threonine-protein phosphatase)   |
| FGSG_05281    | 0.49                                        | FaPpg1 (serine/threonine-protein phosphatase)    |
| FGSG_01464    | 0.70                                        | FaSit4 (serine/threonine-protein phosphatase)    |
| FGSG_08634    | 0.20                                        | FaAreA (nitrogen regulator protein)              |
| FGSG_06977    | 0.30                                        | FaMsg5 (dual specificity phosphatase)            |
| FGSG_10313    | 0.45                                        | FaMgv1 (mitogen-activated protein kinase spm1)    |
| FGSG_09815    | 1.23                                        | FaPp2A (serine/threonine–protein phosphatase)    |

This study shows that rapamycin does not suppress the expression of FaTor RNA and FaFKbp12 RNA (Table 4).

In *S. cerevisiae*, Tap42 (a common regulatory subunit for type2A family members) interacts with the catalytic subunit of type2A protein phosphatases, including Pph3, Pph21, Pph22, Sit4 and Ppg1 and control Tor kinase\textsuperscript{36}. In *F. graminearum*, it is thought that FgPp2A, FgSit4 and FgPpg1 interact with FgTap42\textsuperscript{37}. In this study, low concentration rapamycin treatment reduced the RNA levels of FgTap42, FgPpg1 and FgSit4, but the expression of FgPp2A was not decreased (Table 4). This indicates that low concentration rapamycin partly inhibits the function of FaTor pathway. In *S. cerevisiae* it is known that deletion of Ppg1 has no obvious effect on cell growth\textsuperscript{38}. However, in *F. graminearum*, deficiency of FgPpg1 causes various defects, including reduction of mycelium proliferation and impairment of asexual reproduction and sexual development\textsuperscript{39}. In *F. asiaticum*, the significance of FaPpg1 is still unknown.

Downstream of FaPpg1, there is a global nitrogen regulator, FaAreA. The expression of FaAreA is highly induced when DON is produced\textsuperscript{3}. The rapamycin treatment reduced FaAreA expression in this study. When expression of FaAreA is suppressed, expression of the transcription factor genes, Tri6 and Tri10 is suppressed\textsuperscript{40}, so that DON is not produced.

Within the Tor pathway, FgSit4 and FgPpg1 are known to positively regulate the phosphorylation of FgMgv1 through interaction with FgMsg5, a negative regulator of the cell wall integrity (CWI) pathway\textsuperscript{41}. In *F. graminearum*, the CWI pathway is known to perform a variety of cellular functions such as virulence, mycelial growth, functions including sexual regulation\textsuperscript{42}. Even in *F. asiaticum*, low concentrations of rapamycin suppress the expression of FaMsg5 and FaMgv1 genes.

The FaPp2A gene, which is also thought to interact with FaTap42, was not suppressed by low concentration rapamycin. FaPp2A is a holoenzyme in all eukaryotes, but its activator has many miscellaneous components in filamentous fungi\textsuperscript{38}. This study revealed that expression of FaPp2A is different compared with the other two type 2A protein phosphatase (FaSit4 and FaPpg1) genes after low concentration rapamycin treatment. Further studies are needed to determine how the three protein phosphatases interact with FgTap42 in the FaTor pathway.

**Conclusion**

Low concentration rapamycin inhibits the growth of *F. asiaticum* to 40%, but completely suppresses the production of DON and the expression of DON-related genes. Rapamycin does not suppress the expression of the FaTor gene, but downstream genes are partially suppressed.

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