Sains Malaysiana 54(2)(2025): 461-471
http://doi.org/10.17576/jsm-2025-5402-12

The Global Regulator VeA Modulates ATP Sulfurylase to Mediate Adenosine Synthesis Resulting in Inhibitory Activity of Endophytic *Fusarium solani* against A549 Cells

(Pengawal Selia Global VeA Memodulasi ATP Sulfurilase untuk Mengantara Sintesis Adenosin Menghasilkan Aktiviti Perencatan Endofit *Fusarium solani* terhadap Sel A549)

YULING JIANG[1,2], LU CAI[1,2], QING XIAO[2], GUIHUA LIU[2], RONGFEI LIU[1,2], YAN ZHAO[2], ZHANGJIANG HE,[1,2,*] & JICHUAN KANG[1,2]

[1]College of Pharmacy, Guizhou University, Guiyang, People's Republic of China
[2]Engineering Research Center of the Utilization for Characteristic Bio-Pharmaceutical Resources in Southwest, Ministry of Education, Guizhou University, Guiyang 550025, China

Received: 29 April 2024/Accepted: 18 November 2024

ABSTRACT

Adenosine is an endogenous nucleoside that promotes apoptosis in cancer cells. Overexpression of the global regulator VeA gene (*FsveA*) in *Fusarium solani* resulted in the accumulation of multiple secondary metabolites, including adenosine. In this study, we utilised transcriptome and metabolome data to speculate on the key genes that impact adenosine synthesis in fungi. The c35754.graph_c1 gene encodes an ATP sulfurylase (Fsmet3) that was significantly up-regulated in *F. solani* mutant overexpressing VeA (*FsveA*^{OEV14}). The qRT-PCR results were consistent with the transcriptome data, indicating that *Fsmet3* was regulated by FsveA. Overexpression of *Fsmet3* (*Fsmet3*^{OE}) resulted in a significant increase in adenosine content and the inhibitory activity of the fungi against the human non-small cell lung cancer A549 cells. Moreover, analysis of *Fsmet3*^{OE} metabolome showed that *Fsmet3* not only up-regulated adenosine, but also significantly increased the accumulation of anti-tumor active substances such as resorufin and 13*S*-hydroxydodecanoic acid. Overall, our findings demonstrated that FsveA regulates *Fsmet3* to mediate adenosine synthesis, resulting in potent inhibitory effects against A549 cells by endophytic *F. solani*.

Keywords: Adenosine; ATP sulfurylase; *Fusarium solani*; global regulator VeA; metabolomes

ABSTRAK

Adenosin adalah nukleosida endogen yang menggalakkan apoptosis dalam sel kanser. Pengekspresan melampau gen pengawal atur global VeA (*FsveA*) dalam *Fusarium solani* mengakibatkan pengumpulan pelbagai metabolit sekunder, termasuk adenosin. Dalam kajian ini, kami menggunakan data transkriptom dan metabolom untuk membuat spekulasi mengenai gen utama yang memberi kesan kepada sintesis adenosin dalam kulat. Gen c35754.graph_c1 mengekodkan ATP sulfurilase (Fsmet3) yang dikawal atur menaik dengan signifikan dalam *F. solani* mutan yang mengekspres VeA secara melampau (*FsveA*^{OEV14}). Hasil qRT-PCR didapati tekal dengan data transkriptom yang menunjukkan bahawa *Fsmet3* dikawal atur oleh FsveA. Pengekspresan melampau *Fsmet3* (*Fsmet3*^{OE}) mengakibatkan peningkatan signifikan dalam kandungan adenosin dan aktiviti perencatan kulat terhadap sel kanser paru-paru bukan sel kecil manusia A549. Selain itu, analisis metabolom *Fsmet3*^{OE} menunjukkan bahawa *Fsmet3* bukan sahaja mengawal atur adenosin secara menaik, tetapi juga meningkatkan pengumpulan bahan aktif anti-tumor dengan signifikan seperti resorufin dan asid 13*S*-hidroksidodekanoik. Secara keseluruhan, penemuan kami menunjukkan bahawa FsveA mengawal atur *Fsmet3* untuk memperantara sintesis adenosin, menghasilkan kesan perencatan yang kuat terhadap sel A549 oleh *F. solani* endofit.

Kata kunci: Adenosin; ATP sulfurilase; *Fusarium solani*; metabolom; pengawal atur global VeA

INTRODUCTION

Endophytic fungi are fungi that coexist with plant tissues during specific stages of their life cycle without inducing evident disease symptoms in the plant tissues (Brakhage 2013). They exist in almost all plant tissues, which indicates that they have rich material sources. The unique physiological and metabolic mechanisms enable endophytic fungi to adapt to the special environment inside plants and produce a variety of bioactive substances (Terkar & Borde 2021). In addition, endophytes co-evolve with

their host plants over extended periods of time, and possess the capability to produce metabolites that share similar or identical medicinal properties as those found in the host plants. Endophytic fungi can even help the medicinal host plants synthesise effective active compounds. This breakthrough discovery provides a new method for isolating effective compounds with similar effects to natural drugs directly from plant tissues (Ancheeva, Daletos & Proksch 2020). Alkaloids, steroids, terpenes, anthraquinones, cyclic peptides, and flavonoids represent key metabolites that have been recently identified from endophytic fungi (Vasundhara, Reddy & Kumar 2019). Various endophytes have the capability to generate secondary metabolites with antitumor, antibacterial, anti-inflammatory, antiviral, antifungal, and other biological activities, so the diversity of secondary metabolites from endophytic fungi has great potential for new drug development (Fadiji & Babalola 2020).

Intracellular adenosine signaling mechanisms can occur independently of adenosine receptor interactions. Adenosine is transported across cells by adenosine transporters. When it enters cells, it is converted to adenosine phosphate (AMP) by adenosine kinase. This process activates AMP-activated protein kinase (AMPK), which regulates energy homeostasis by regulating ATP levels (Aymerich et al. 2006). Adenosine-activated AMPK plays a crucial role in regulating numerous cellular processes such as cell migration, apoptosis, and proliferation. Additionally, studies have shown that AMPK negatively regulates aerobic glycolysis and inhibit tumor growth (Faubert et al. 2013; Patel et al. 2015). Thus, the anticancer properties of adenosine may be mediated through adenosine receptors dependent and independent pathways, resulting in immunosuppressive responses characterised by decreased proliferation, diminished effector functions, and decreased cytokine secretion (Virtanen et al. 2014). Intracellular transport of adenosine has been shown to act through AMP to cause up-regulation of p53 expression and induce apoptosis (Nogi et al. 2012). Recent studies on adenosine and its receptors have garnered great attention in the field of cancer pathology, so how to improve adenosine production attracted our attention.

Continuous and comprehensive investigation of endophytic fungi has shown that the number of identified secondary metabolites in these fungi is significantly lower than the number of secondary metabolism gene clusters (Alam et al. 2021). Majority of the secondary metabolism gene clusters exhibit minimal or no gene expression, resulting in undetectable level of corresponding metabolite production. The secondary metabolic pathway of endophytic fungi is a complex regulatory process, including transcription, translation and phenotypic inheritance (Bills & Gloer 2016). It is not only regulated by transcriptional activation and gene expression of gene clusters, but also by pathway-specific regulatory factors and multiple regulation of global transcription factors such as LaeA, VeA, and McrA (Feng, Xing & Hu 2011).

Fusarium solani is a phytopathogenic fungus with a rotting effect on plant roots (Mohd et al. 2023) and it is also an animal pathogen (Chai et al. 2023). The vast majority of the secondary metabolites it produces are biologically active, including the common cyclic peptides and enols, naphthoquinones, and the active sesquiterpene epoxides (Jiao et al. 2015). Our research group isolated an endophytic fungus, *F. solani*, from the stem of the medicinal plant *Nothapodytes pittosporoides* (Oliv.) Sleum, strain number HB1-J1, and found that the ethyl acetate layer of the crude extract of the fermented strain had an inhibitory effect on human non-small cell lung cancer A549 cells.

Based on this, *FsveA* was overexpressed in *F. solani* and the mutant *FsveA*^{OEV14} was successfully obtained (Cai et al. 2022). It was found that the metabolism of the mutant *FsveA*^{OEV14} was changed, and the antitumor activity was significantly increased compared with that of the wild-type strain. Metabolomic analysis of mutant strain *FsveA*^{OEV14} showed a substantial enrichment of various metabolites in the purine metabolism pathway, with a notable up-regulation in adenosine content. With the assistance of transcriptome analysis, it was observed that the *Fsmet3* gene showed the most significant up-regulation compared to the wild type. We hypothesise that *Fsmet3* is a key gene whose overexpression affects the production of the secondary metabolite adenosine. The aim of this study was to investigate the molecular basis of FsveA-mediated adenosine production by the *Fsmet3* gene in wild type *F. solani* HB1-J1.

MATERIALS AND METHODS

ISOLATION OF *Fsmet3*

A strain HB1-J1 was isolated from the stem of the medicinal plant *N. pittosporoides* and identified as endophytic *F. solani*. The *Met3* gene of *F. oxysporum* was utilised as a template, with *Met3*-F and *Met3*-R primers employed to amplify the ORF framework of the *Met3* gene for isolation of the *Fsmet3* gene (Table 1). Subsequently, Basic Local Alignment Search Tool online search on the NCBI website was conducted to analyse the *Met3* gene, followed by multiple sequence alignment analyses using Espript3 online software to align amino acid sequences of *Met3* genes from different species in NCBI.

CONSTRUCTION OF GENE TRANSFORMATION SYSTEM

The *Fsmet3* gene was overexpressed by the PEG-mediated protoplast method. The *Fsmet3* gene was amplified from *F. solani* DNA using *Fsmet3*-F and *Fsmet3*-R as primers. The GPDA-F and hyg-up-R primer pair was used to amplify the upper fragment of GDPA-*hyg* using the pK2*hyg* carrier plasmid as the template. The lower fragment of *hyg* was amplified using hyg-down-F and hyg-down-R primers, and the lower fragment of *hyg* was fused with the *Fsmet3* gene fragment by PCR procedure. Specific primers are shown in

TABLE 1. Primer sequences used in this study

Primer name	Primer sequence (5′-3′)
Fsmet3-F	ATGGCCAACACTCCTCACGGT
Fsmet3-R	TTACAGGCGGTCAAGGAGA
qPCR-Fsmet3 - F	CGACGCCGGTGAAGTTCTTA
qPCR-Fsmet3 - R	AGTACTCGAGAGGGGTAGCG
GPDA-F	TGCAAAGCATGCGGAGAGA
hyg-up-R	CTATTCCTTTGCCCTCGGAC
hyg-down-F	GTATCACTGGCAAACTGTGATGGACG
hyg-down-R	TGAGGAGCGTTGGCCATCTATTCCTTTGCCCTC
Fsmet3-hind-F	GAGGGCAAAGGAATAGATGGCCAACGCTCCTCA
Fsmet3-hind-R	TTACAGGCGGTCGAG

Table 1. Protoplasts were obtained by enzymatic hydrolysis of *F. solani*'s mycelia and mixed with the upper fragment of GPDA-hyg and the lower fragment of *hyg-Fsmet3* to be transformed under certain concentrations of $CaCl_2$ and PEG. The protoplasts were induced by PEG to promote the absorption of the two fragments, and calcium ions were used to increase membrane permeability, and the culture was shaken overnight. TB3 culture medium was added to an inverted plate and cultured inverted at 25 °C for 24 h. When the culture temperature of TB3 was appropriate, ampicillin and the corresponding concentration of hygromycin were added to the upper plate and cultured at 25 °C for 1-5 days. The transformants grown on the double-layer plate were selected and placed on the PDA plate containing hygromycin for verification screening (Cai et al. 2020).

REAL-TIME QUANTITATIVE PCR

Mycelia of WT and *Fsmet3*^{OE} were collected after 7 days of fermentation in 1/4 SDB medium. Total RNA quality and concentration were determined, followed by DNA removal after extraction. cDNA was synthesised using the GenStar kit (GenStar, Beijing, China) and used as a template for real-time quantitative PCR (RT-qPCR) analysis (Bio-Rad, Harkles, CA, USA) with a two-step amplification method. *Fusarium β-tubulin* gene was used as the reference gene. The results were calculated according to the $2^{-\Delta\Delta T}$ method to analyse the expression level of the target gene for screening (Alexander, McCormick & Blackburn 2008). The experiment was repeated three times under identical experimental conditions using the RT-qPCR primers shown in Table 1.

PREPARATION OF STRAIN CRUDE EXTRACT

The overexpression mutant *FsveA*^{OEV14} and wild-type HB1-J1 strains were cultured on PDA plates for 5 days and then punched and inoculated into maize liquid medium for 5 days. The spore suspension was prepared with 0.05% (v/v) Tween-80, and the concentration was adjusted to 1×10^5 conidia/mL using a blood cell counting plate. The spore suspension was inoculated into sand liquid medium (500 mL/bottle) and fermented at 28 °C and 180 rpm for 7 days. The fermented mutant and WT strain were filtered through liquid culture, the mycelium was separated from the bacterial solution, and the crude extract was extracted with ethyl acetate (EtOAc) three times. The crude extract was obtained after decompression and concentration and stored at 4 °C, which was convenient for subsequent HPLC analysis, anti-tumor activity, and metabolomics detection. The metabolome was analysed using the biodeep programme (https://www.biodeep.cn).

HPLC ANALYSIS

The fermentation broth of *Fsmet3*^{OE19} overexpression mutant and HB1-J1 wild type strain was concentrated with ethyl acetate under reduced pressure to obtain the crude extract. The crude extract (0.1 g) was weighed precisely and mixed with 1 mL of 50% methanol to obtain the test solution. Adenosine standard (McLlin, Shanghai, China) was dissolved in methanol solution (50% concentration) to form a control solution containing 6.40 μg/mL of adenosine, and the injection volume was 20 μL using Agilent Zorbax SB-C18 column (4.6 mm×150 mm, 5 μm; mobile phases: acetonitrile (A) and 0.1% phosphoric acid (B); detection wavelength: 254 nm; column temperature: 40 °C; flow rate: 1.0 mL/min) (Fu, Chen & Cao 2018).

ANTI-TUMOR ACTIVITY TEST

It has been reported in the literature that the crude extract of the ethyl acetate layer of *F. solani* had a significant inhibitory effect on A549 cells compared to the negative control, and the inhibitory effect was enhanced with the

increase of the concentration of the crude extract, which was 37.87% at the concentration of 300 μg·mL-1, with an IC_{50} value of 362.21 ± 1.34 μg/mL (Cai et al. 2022).

Lung cancer A549 cells (Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences) in logarithmic growth phase were adjusted to a concentration of 1×10^5 cells/mL. Then, 100 μL of cell suspension was added into each well of a 96-well plate. Meanwhile, the blank group and normal control group were incubated at 37 °C with 5% CO_2 overnight until the cell monolayer covered more than 70% of the bottom of the wells. The positive drug adriamycin and crude extract were dissolved and filtered in dimethyl sulfoxide (DMSO), followed by treatment of the cells with different concentrations gradients of adriamycin and crude extract for a duration of 24 h (Dehghan et al. 2022). MTT solution (20 μL) was added to each well and incubated at 37 °C for 4 h. Then, DMSO (150 μL) was added to dissolve the Kazan crystals formed by living cells, and the absorbance OD value was measured at 490 nm using a microplate reader (BIOBRI, Chengdu, China).

STATISTICAL ANALYSIS

All data were the results of three repeated experiments. Statistical analysis was performed using GraphPad Prism version 8 (GraphPad Software, San Diego, CA, USA) and T-test.

RESULTS

Fsvea REGULATES *Fsmet3* TRANSCRIPTION

Following the overexpression of the global regulatory factor FsveA in *F. solani*, the transformed strain *FsveA*^{OEV14} was successfully obtained. In comparison to the HB1-J1 wild type strain, the crude extract of the overexpressed strain exhibited significantly enhanced anti-tumor activity, with 14.69% increment in inhibition rate and an apoptosis ratio approximately 4.86 times higher than that of the wild strain (Cai et al. 2022). Notably, significant differences in metabolite yield were observed between the overexpressed strain *FsveA*^{OEV14} and the WT strain. To elucidate FsveA-mediated metabolism at a molecular level, metabolomics and transcriptomic analyses were conducted on crude extracts from both wild type *F. solani* and *FsveA*^{OEV14} strains. An LC/MS non-targeted metabolomics method was used to detect metabolites in the WT and *FsveA*^{OEV14} strains. The data showed significant metabolic differences between the two strains. The differential metabolites were analysed under the conditions of FC > 1, P < 0.05, and VIP > 1, resulting in 304 up-regulated and 169 down-regulated metabolites. Combining metabolic and transcriptome data analysis, it was found that 8 metabolites were significantly up-regulated in purine metabolism (ko00230), and the difference ratio of adenosine metabolite was about 2.91 times (log2FC=1.54). There were 11 differentially expressed genes up-regulated at the transcriptional level, among which the *Fsmet3* gene (ID: c35754.graph_c1) exhibited the largest up-regulation (Table 2). Therefore, we speculated that the *Fsmet3* gene may be the key gene in *FsveA*, mediating the up-regulation of secondary metabolites in the purine metabolism of the *FsveA*^{OEV14} strain. The *Fsmet3* gene encodes ATP:sulfate adenylyltransferase (EC 2.7.7.4) also known as ATP sulfurylase, which is the first enzyme in the sulfate assimilation pathway. This enzyme activates inorganic sulfate by adenylation of ATP, forming 5'-adenylyl sulfate (APS) and inorganic pyrophosphate. ATP thioacylase is widely presented in plant chloroplasts and cytoplasm (Hatzfeld et al. 2000).

TABLE 2. A statistical table of 11 key genes up-regulated in purine metabolism

ID	Log_2FC	Regulated
c35754.graph_c1	3.22	up
c35838.graph_c0	1.84	up
c30437.graph_c0	1.79	up
c35915.graph_c1	1.68	up
c22607.graph_c0	1.63	up
c23535.graph_c0	1.52	up
c29333.graph_c0	1.44	up
c34390.graph_c0	1.30	up
c29333.graph_c1	1.21	up
c32950.graph_c1	1.17	up
c29260.graph_c2	1.17	up

ISOLATION OF *Fsmet3* GENE

The homologous gene *Fsmet3* in the strain was amplified and sequenced using the *F. solani* genome as a template. The sequencing results were analysed using the BLAST online search on the NCBI website. Homologous sequence comparison analysis using the online software Espript3 showed that the Met3 protein had more than 85% homology with other strains, and this gene was named *Fsmet3* (Figure 1(a)).

Fsmet3 MEDIATES SECONDARY METABOLISM OF *F. solani*

The *Fsmet3* gene was significantly up-regulated in the transcriptomic data of the *FsveA*^{OEV14} strain. qRT-PCR was used to analyse the transcription levels of the *Fsmet3* gene in the wild-type and *FsveA*^{OEV14} strains to further verify the relationship between the *Fsmet3* and *FsveA* genes. The results showed that *FsveA* overexpression could significantly up-regulate *Fsmet3* gene transcription (Figure 1(b)). On this basis, we overexpressed the *Fsmet3* gene by using the principle of protoplast two-fragment fusion and successfully obtained a *Fsmet3*^{OE19} mutant with expression of the *Fsmet3* gene more than 7 times higher than the wild type via resistance screening and qRT-PCR analysis (Figure 1(c), 1(d), 1(e), 1(f)). Wild-type and *Fsmet3*^{OE19} mutant strains were fermented at the same time, and the ethyl acetate layer of their crude extract was analysed by HPLC to detect the content of the secondary metabolite adenosine.

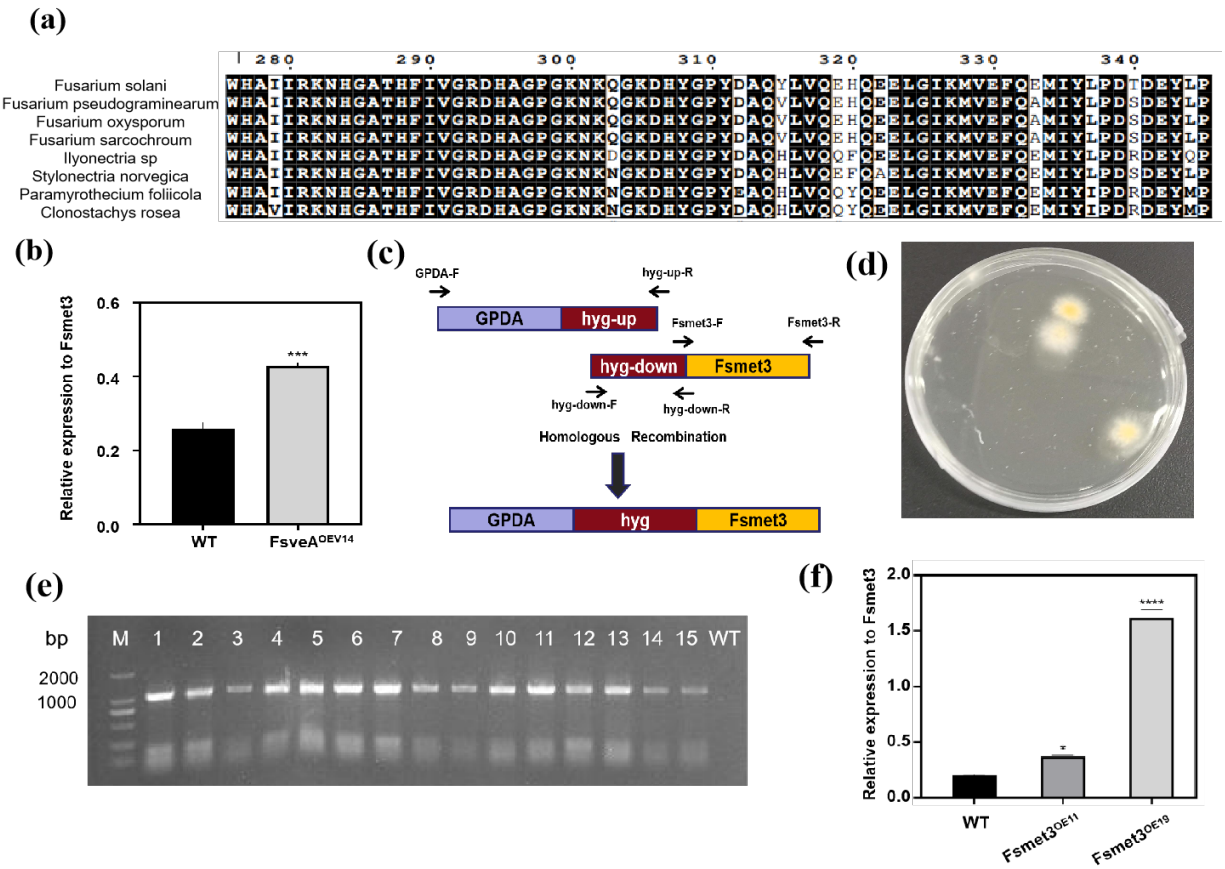

FIGURE 1. (a) Protein sequence alignment of Fsmet3, (b) *Fusarium solani* (wild-type) and *FsveA*^{OEV14} strains were cultured in a 1/4 SDB medium solution for 7 days, and *Fsmet3* gene expression was measured by RT-qPCR, (c) Schematic representation of the protoplast double fragments, (d) Transformant status on the upper plate of TB3 medium on the third day, (e) PCR validation of hygromycin resistance genes (*hyg*) on transformants on resistance plates, and (f) *Fsmet3* gene expression was detected by RT-qPCR after *F. solani* (wild-type) and *Fsmet3* gene overexpression strains were grown in 1/4 SDB medium for 7 days. Bar graphs are shown as the mean statistical analysis of three biological replicates. The experiment was repeated three times, the average was taken, the error bar is the standard deviation ($\pm$ SD). *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, ****$p < 0.001$

The results showed that the content of adenosine in mutant strains increased significantly after overexpression of the *Fsmet3* and *FsveA* genes, while the *FsveA*^{OEV14} strain had the highest content (Figure 2(a), 2(b)).

In order to explore the material basis of *Fsmet3*-mediated metabolism, the differential metabolites between WT and *Fsmet3*^{OE19} strains were analysed by LC/MS non-targeted metabolomics. Principal component analysis showed significant separation between the WT and *Fsmet3*^{OE19} strain samples. The differential metabolite cluster heat map (Figure 3(a)) showed that *Fsmet3*^{OE19} and WT repeatedly formed independent branches and showed significant metabolic differences. According to the aforementioned data, significant disparities in secondary metabolites were observed between the wild type strain and the overexpression strain. Analysis of differential metabolites under conditions FC > 1, F < 0.05, and VIP > 1 showed co-expression of 362 metabolites, with 171 being differentially expressed and 191 showing no differential expression (Figure 3(b)). Further enrichment analysis using KEGG pathways demonstrated that the differentially expressed metabolites were enriched in various biosynthetic pathways including ABC transporters, linoleic acid metabolism, pyrimidine metabolism, histidine metabolism, as well as several other secondary metabolite pathways (Figure 3(c)). Subsequently, a total of 98 compounds exhibiting up-regulation under conditions $\log_2$FC > 0.5, P value < 0.05 and VIP >1 were identified among the screened differential metabolites; from these compounds, we selected the top ten up-regulated ones (Table 3). The *Fsmet3* gene is a nucleotidyltransferase, and in *Burkholderia pseudomallei,* nucleotidyltransferase (BpHldC) is involved in the heptose biosynthesis pathway affecting lipopolysaccharide production (Park et al. 2018). These findings suggested that besides mediating adenosine production, overexpression of *Fsmet3* gene also influences the production of other secondary metabolites in this strain.

Fsmet3 MEDIATED THE ANTITUMOR ACTIVITY OF THE STRAIN

MTT assay was used to detect the anti-tumor activity of crude extracts of wild-type and *FsveA*^{OEV14} strains on A549 cells. After overexpression of *Fsmet3* gene, the anti-tumor activity of the mutant strain was significantly increased, and the semi-inhibitory concentration of the strain decreased from 462.7 μg/mL to 285.2 μg/mL (Figure 4). After the overexpression of *Fsmet3* gene, the content of adenosine in the crude extract of *Fsmet3*^{OE19} strain was higher than that of WT strain, and the inhibition rate of A549 cells was also increased.

Through metabolomics analysis, a variety of active anti-tumor substances were screened. Metabolomic analysis of *Fsmet3*^{OE19} crude extract was performed. A variety of active anti-tumor substances were screened by metabolomics analysis. Metabolomics analysis of the *Fsmet3*^{OE19} crude extract was performed. Anti-tumor substances such as luteolin (Rakoczy et al. 2023), p-hydroxycinnamic acid (Ferreira et al. 2019), 13*S*-hydroxydodecanoic acid (Li et al. 2015), resorcinol (Pamu et al. 2012), linoleic acid (Dachev et al. 2021), caffeic acid (Kabała-Dzik et al. 2018), malic acid (Deng et al. 2020), 3-methylthiopropionic acid (Nakamura et al. 2008), campesterol (Bae et al. 2021), and indole-3-methyl acetate (Zhang et al. 2006) were

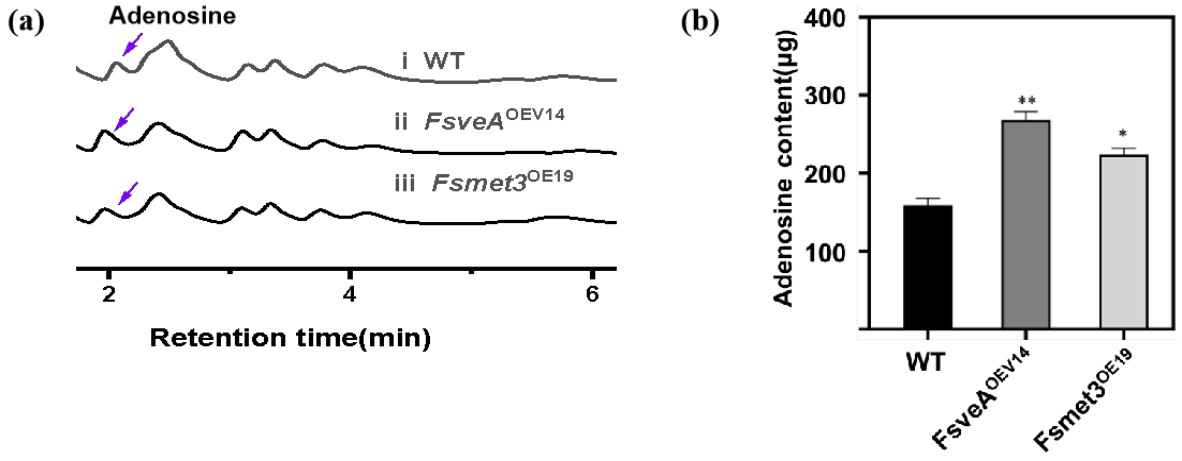

FIGURE 2. (a) HPLC analysis of adenosine, a secondary metabolite of wild-type, *FsveA*^{OEV14}, and *Fsmet3* strains. The blue arrow points to the peak out position of adenosine, (b) Comparison of adenosine content after HPLC analysis of the initial extracts of WT, *FsveA*^{OE14}, and *Fsmet3*^{OE19} strains. The experiment was repeated three times, the results were averaged, and the error bar is the standard deviation (± SD). $^{*}p < 0.05$, $^{**}p < 0.01$

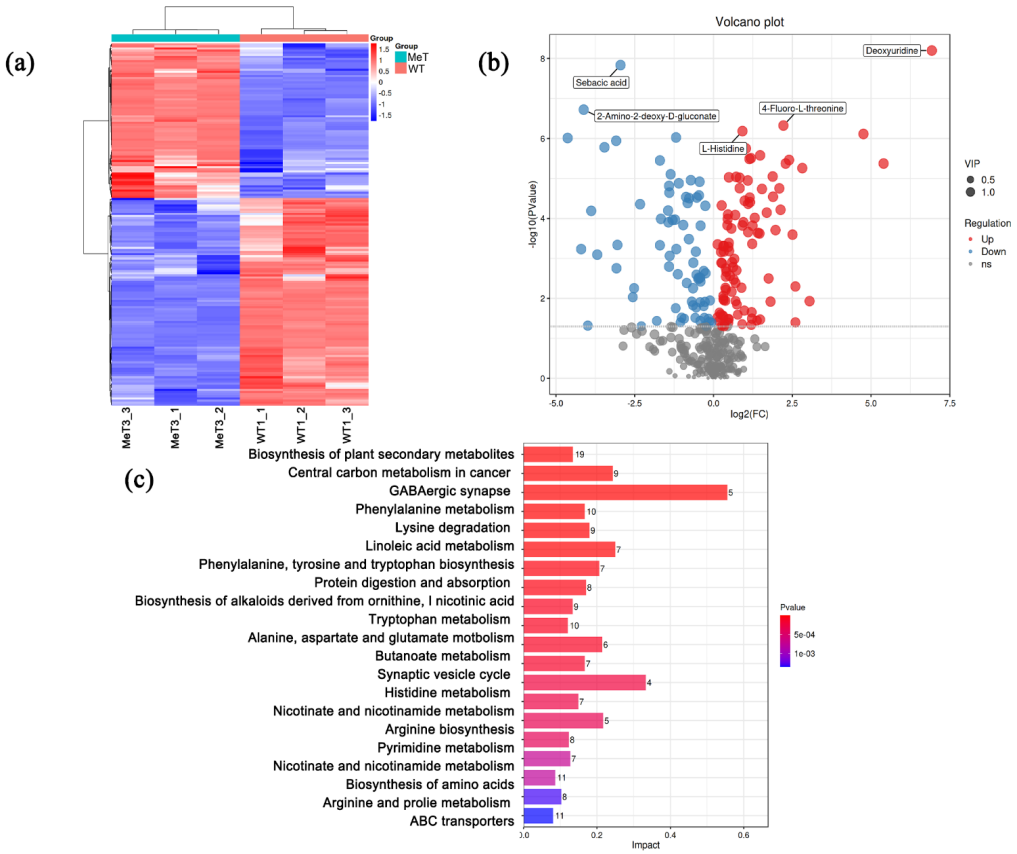

FIGURE 3. Classification of differential metabolites, (a) Heat map of differential metabolites clustering, (b) Volcano map of differential metabolites, and (c) Bar graph of metabolic pathway influencing factors

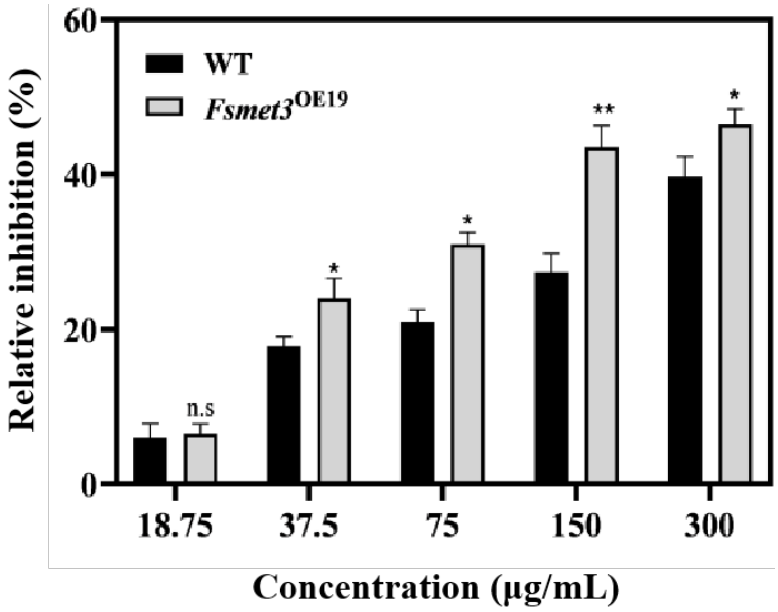

FIGURE 4. MTT assay was used to determine the relative inhibition rate of crude extracts of WT and *Fsmet3*^{OE19} strains on A549 cells. The experiment was repeated three times, the results were averaged, and the error bar is the standard deviation (±SD). *$p < 0.05$, **$p < 0.01$

up-regulated. Among them, the flavonoid luteolin was up-regulated most significantly (Table 4). These results indicated that the *Fsmet3* gene not only mediated the production of adenosine but also affected the production of other anti-tumor substances in *F. solani*, which showed that the *Fsmet3* gene was the key gene to regulate the production of tumor suppressor adenosine and mediate the anti-tumor activity of *F. solani*.

DISCUSSION

Secondary metabolites (SMs) are low-molecular-weight compounds produced by primary metabolism (Yu et al. 2023). Although endophytic fungi have great potential to produce special and abundant SMs, it is difficult to achieve rational needs and targeted synthesis of target compounds due to genetic manipulation, culture conditions, detection methods, and other restrictions. In traditional biological research laboratory settings, the biosynthetic gene clusters responsible for secondary metabolites in endophytic fungi are typically inactive. These silent gene clusters often regulate abundant secondary metabolism resources, resulting in low or no secondary metabolic yield (Tomm, Ucciferri & Ross 2019). In our previous study, endophytic *F. solani* HB1-J1 was isolated from the medicinal plant *N. pittosporoides* and its crude extract showed good inhibitory activity against A549 cells after biological activity screening. In order to further analyse the material and molecular basis of the anti-tumor activity of the strain, we overexpressed the global regulator VeA in the *F. solani* strain and successfully obtained a highly overexpressed strain *FsveA*^{OEV14}. The anti-tumor activity of the crude extract was significantly up-regulated compared with that of the wild type. The differential metabolites were found to be notably enriched in ABC transporters, purine metabolism, phenylalanine metabolism, 2-oxo-carboxylic acid metabolism, amino-acetyl-tRNA biosynthesis, and indole alkaloid metabolism. Combined metabolomics and transcriptome analysis of the *FsveA*^{OEV14} strain showed that adenosine content in the purine metabolism

TABLE 3. Top ten secondary metabolic compounds up-regulated in the metabolomics analysis

Name	Log_2FC	VIP	Regulated
Deoxyuridine	6.93	1.36	up
Luteolin	5.4	1.38	up
6-Hydroxyhexanoic acid	4.76	1.37	up
4-Hydroxycinnamic acid	3.05	1.33	up
N6-Acetyl-L-lysine	2.82	1.36	up
13S-hydroxyoctadecadienoic acid	2.6	1.25	up
9,10-Epoxyoctadecenoic acid	2.6	1.29	up
Agmatine	2.51	1.34	up
1,3-Benzenediol	2.4	1.37	up
Linoleic acid	2.29	1.36	up

TABLE 4. Antitumor compounds up-regulated in metabolomics analysis

Name	Log_2FC	VIP	Regulated
Luteolin	5.4	1.36	up
4-Hydroxycinnamic acid	3.05	1.32	up
13S-hydroxyoctadecadienoic acid	2.6	1.35	up
1,3-Benzenediol	2.4	1.36	up
Linoleic acid	2.29	1.35	up
Caffeic acid	2.13	1.36	up
Maslinic acid	1.97	1.34	up
3-Methylthiopropionic acid	1.89	1.36	up
Campesterol	1.81	1.32	up
Methyl (indol-3-yl) acetate	1.48	1.35	up

pathway was significantly up-regulated. Combined with transcriptome data analysis, the *Fsmet3* gene encoding the ATP sulfurylase enzyme was the most up-regulated in purine metabolism. We suspected that the up-regulation of the *Fsmet3* gene may mediate the production of adenosine. To confirm the relationship between the *FsveA*^{OEV14} strain and the *Fsmet3* gene, the transcript levels of *Fsmet3* in wild-type and *FsveA*^{OEV14} were analysed by qRT-PCR. The results showed that overexpression of *FsveA* resulted in a substantial up-regulation of *Fsmet3* transcript levels.

After resistance screening and qRT-PCR analysis, we successfully obtained a *Fsmet3*^{OE19} mutant whose *Fsmet3* gene expression was 7 times higher than that of the wild type. The wild-type and *Fsmet3*^{OE19} mutant strains were simultaneously fermented, and the content of adenosine in the ethyl acetate layer of the crude extract was analysed by HPLC. The results showed that the yield of adenosine was significantly increased when the *Fsmet3* and *FsveA* genes were overexpressed, and the *FsveA*^{OEV14} strain had the highest content. The MTT assay was used to detect the anti-tumor activity of the wild-type and *FsveA*^{OEV14} strain crude extracts on A549 cells. The anti-tumor activity of the strain increased with overexpression of the *Fsmet3* gene, and the IC_{50} value of the strain decreased from 462.7 μg/mL to 285.2 μg/mL. Metabolomics analysis of *Fsmet3*^{OE19} crude extract showed that anti-tumor substances such as luteolin, p-hydroxycinnamic acid, and 13*S*-hydroxylauric acid were up-regulated, among which flavonoid luteolin was the most significantly up-regulated.

The *Fsmet3*^{OE19} metabolome data were screened for the top ten differential metabolites with the largest up-regulation folds and analysed in conjunction with the *FsveA*^{OEV14} metabolome (Table 5). The most up-regulated metabolite was the nucleoside deoxyuridine, which was also up-regulated in the *FsveA*^{OEV14} metabolome. Most of the top ten differential metabolites in the *FsveA*^{OEV14} metabolome were up-regulated in the *FsveA*^{OEV14} metabolome, which also included the secondary metabolites with antitumour activity, luteolin, 4-hydroxycinnamic acid, 1,3-benzenediol, and linoleic acid, whereas 6-hydroxyhexanoic acid was up-regulated only in the *Fsmet3*^{OE19} metabolome, with no difference in the *FsveA*^{OEV14} VeA, as a fungus-specific global regulator, can activate silent gene clusters within the fungus, resulting in higher levels of transcription and expression of its secondary metabolites (Lyu et al. 2020). The overexpression of *FsveA* affected the transcriptional level of *Fsmet3*, thereby affected the production of secondary metabolites. Whereas FsveA mediates the transcriptional level of *Fsmet3*, so the trend of affecting secondary products is consistent.

After overexpression of the *Fsmet3* gene, the adenosine content in the crude extract of strain *Fsmet3*^{OE19} increased compared with that of the WT strain, and the inhibitory rate on A549 cells also increased significantly. A variety of anti-tumor active substances were screened out through metabolomics analysis, indicating that the *Fsmet3* gene not only mediates adenosine production but also affects the production of other anti-tumor substances, showing that the global regulatory factor VeA mediates the anti-tumor active substance of *F. solani* with the *Fsmet3* gene as the key gene to regulate the production of adenosine. It also mediated the anti-tumor activity of *F. solani*. It is speculated that the overexpression of the *VeA* gene may affect the biosynthesis of secondary metabolic anti-tumor substances via the up-regulation of several genes, which leads to the difference in anti-tumor activity between *FsveA*^{OEV14} and WT strain crude extracts. The production of secondary metabolites can be improved by further studying the molecular regulation mechanism of secondary metabolism in fungi and exploring the key genes (Grau et al. 2018).

TABLE 5. Comparison of the top 10 differential secondary metabolites of up-regulated multiplicity in the WT and *Fsmet3*^{OE19} metabolomes with data from the WT and *FsveA*^{OEV14} metabolomes

Metabolites	Log_2FC	The Log2FC of this compound in the WT and *FsveA*^{OEV14} metabolomes
Deoxyuridine	6.93	0.74
Luteolin	5.4	1.2
6-Hydroxyhexanoic acid	4.76	NO
4-Hydroxycinnamic acid	3.05	0.36
N6-Acetyl-L-lysine	2.82	0.81
13S-hydroxyoctadecadienoic acid	2.6	0.97
9,10-Epoxyoctadecenoic acid	2.51	0.21
Agmatine	2.4	1.61
1,3-Benzenediol	2.6	0.57
Linoleic acid	2.29	1.24

In this study, we identified a *Fsmet3* gene encoding ATP sulfurylase and transformed it using a protoplast transformation method, which significantly enhanced the transformation efficiency of *Fusarium* spp. Overexpression of *Fsmet3* effectively facilitated the biosynthesis of adenosine as a metabolite. The crude extract from the mutant strain exhibited augmented inhibitory effects on A549 cells and demonstrated increased production of other secondary metabolites with potential antitumor activity, as shown by metabolomics analysis. This study presents novel insights into enhancing secondary metabolite production in *F. solani*.

ACKNOWLEDGEMENTS

We would like to thank the Science Foundation of China (Nos. 32160667, 31901947, and 32170019), Qian Kehe-ZK [2021]-145/the BasicProject of Guizhou Provincial Natural Science Foundation, Gui Da Ren Ji He Zi (2019) 71/Guizhou University PhD Fund Project, and Guizhou University Incubation [2019] 19/Guizhou University Incubation Program for their financial assistance.

REFERENCES

Alam, B., Lǐ, J., Gě, Q., Khan, M.A., Gōng, J., Mehmood, S., Yuán, Y. & Gǒng, W. 2021. Endophytic fungi: From symbiosis to secondary metabolite communications or vice versa? *Frontiers in Plant Science* 12: 791033.

Alexander, N.J., McCormick, S.P. & Blackburn, J.A. 2008. Effects of xanthotoxin treatment on trichothecene production in *Fusarium sporotrichioides*. *Canadian Journal of Microbiology* 54(12): 1023-1031.

Ancheeva, E., Daletos, G. & Proksch, P. 2020. Bioactive secondary metabolites from endophytic fungi. *Current Medicinal Chemistry* 27(11): 1836-1854.

Aymerich, I., Foufelle, F., Ferré, P., Casado, F.J. & Pastor-Anglada, M. 2006. Extracellular adenosine activates AMP-dependent protein kinase (AMPK). *Journal of Cell Scienc*e 119(8): 1612-1621.

Bae, H., Park, S., Yang, C., Song, G. & Lim, W. 2021. Disruption of endoplasmic reticulum and ROS production in human ovarian cancer by campesterol. *Antioxidants* 10(3): 379.

Bills, G.F. & Gloer, J.B. 2016. Biologically active secondary metabolites from the fungi. *Microbiology Spectrum* 4(6): 10.

Brakhage, A.A. 2013. Regulation of fungal secondary metabolism. *Nature Reviews Microbiology* 11(1): 21-32.

Cai, E-P., Mei, D., Zhang, X-M., Sun, X., Li, L-Y., Wu, R-R., Deng, Y-Z., Jiang, Z-D. & Chang, C-Q. 2020. A gene knockout method based on protoplast transformation with two PCR fragments in *Sporisorium scitamineum*. *Mycosystema* 39(12): 2314-2327.

Cai, L., Wang, J., Li, Y., Qin, M., Yin, X., He, Z. & Kang, J. 2022. Global regulatory factor VeA upregulates the production of antitumor substances in endophytic *Fusarium solani*. *Antonie Van Leeuwenhoek* 115(8): 1085-1100.

Chai, C.X., Samat, A., Mohd-Taib, F.S., Bharudin, I. & Tim, J. 2023. Fungal infection of sea turtle eggs in the sea turtle hatcheries in Peninsular Malaysia. *Fungal Ecology* 63: 101243.

Dachev, M., Bryndová, J., Jakubek, M., Moučka, Z. & Urban, M. 2021. The effects of conjugated linoleic acids on cancer. *Processes* 9(3): 454.

Dehghan, R., Beig Parikhani, A., Zeinali, S., Shokrgozar, M., Amanzadeh, A., Ajdary, S., Ahangari Cohan, R., Talebkhan, Y. & Behdani, M. 2022. Efficacy and antitumor activity of a mutant type of interleukin 2. *Scientific Reports* 12(1)：5376.

Deng, J., Wang, H., Mu, X., He, X., Zhao, F. & Meng, Q. 2020. Advances in research on the preparation and biological activity of maslinic acid. *Mini-Reviews in Medicinal Chemistry* 21(1): 79-89.

Fadiji, A.E. & Babalola, O.O. 2020. Elucidating mechanisms of endophytes used in plant protection and other bioactivities with multifunctional prospects. *Frontiers in Bioengineering and Biotechnology* 8: 467.

Faubert, B., Boily, G., Izreig, S., Griss, T., Samborska, B., Dong, Z., Dupuy, F., Chambers, C., Fuerth, B.J., Viollet, B., Mamer, O.A., Avizonis, D., DeBerardinis, R.J., Siegel, P.M. & Jones, R.G. 2013. AMPK is a negative regulator of the Warburg effect and suppresses tumor growth *in vivo*. *Cell Metabolism* 17(1): 113-124.

Feng, H., Xing, W. & Hu, C. 2011. Research advances in global regulator LaeA of filamentous fungi-A review (Article in Chinese). *Wei Sheng Wu Xue Bao* 51(9): 1141-1145.

Ferreira, P.S., Victorelli, F.D., Fonseca-Santos, B. & Chorilli, M. 2019. A review of analytical methods for p-coumaric acid in plant-based products, beverages, and biological matrices. *Critical Reviews in Analytical Chemistry* 49(1): 21-31.

Fu, F., Chen, L. & Cao, Q. 2018. Separation and determination of cordycepin and adenosine in artificial *Cordyceps sinensis* mycelium by gradient UPLC. *IOP Conference Series: Materials Science and Engineering* 394: 022019.

Grau, M.F., Entwistle, R., Chiang, Y-M., Ahuja, M., Oakley, C.E., Akashi, T., Wang, C.C.C., Todd, R.B. & Oakley, B.R. 2018. Hybrid transcription factor engineering activates the silent secondary metabolite gene cluster for (+)-asperlin in *Aspergillus nidulans*. *ACS Chemical Biology* 13(11): 3193-3205.

Hatzfeld, Y., Lee, S., Lee, M., Leustek, T. & Saito, K. 2000. Functional characterization of a gene encoding a fourth ATP sulfurylase isoform from *Arabidopsis thaliana*. *Gene* 248(1): 51-58.

Jiao, X., Lu, X., Chen, A.J., Luo, Y., Hao, J.J. & Gao, W. 2015. Effects of *Fusarium solani* and *F. oxysporum* infection on the metabolism of ginsenosides in American ginseng roots. *Molecules* 20(6): 10535-10552.

Kabała-Dzik, A., Rzepecka-Stojko, A., Kubina, R., Wojtyczka, R.D., Buszman, E. & Stojko, J. 2018. Caffeic acid versus caffeic acid phenethyl ester in the treatment of breast cancer MCF-7 cells: Migration rate inhibition. *Integrative Cancer Therapies* 17(4): 1247-1259.

Li, M-Y., Yuan, H-L., Ko, F.W.S., Wu, B., Long, X., Du, J., Wu, J., Ng, C.S.H., Wan, I.Y.P., Mok, T.S.K., Hui, D.S.C., Underwood, M.J. & Chen, G.G. 2015. Antineoplastic effects of 15(S)-hydroxyeicosatetraenoic acid and 13-S-hydroxyoctadecadienoic acid in non-small cell lung cancer. *Cancer* 121(Suppl 17): 3130-3145.

Lyu, H-N., Liu, H-W., Keller, N.P. & Yin, W-B. 2020. Harnessing diverse transcriptional regulators for natural product discovery in fungi. *Natural Product Reports* 37(1): 6-16.

Mohd, M.H., Nor, N.M.I.M., Azuddin, N.F. & Zakaria, L. 2023. Mycotoxin production by *Fusarium proliferatum* and *Fusarium fujikuroi* causing stem rot of *Hylocereus polyrhizus* in Malaysia. *Malaysian Applied Biology* 52(3): 13-22.

Nakamura, Y., Nakayama, Y., Ando, H., Tanaka, A., Matsuo, T., Okamoto, S., Upham, B.L., Chang, C-C., Trosko, J.E., Park, E.Y. & Sato, K. 2008. 3-Methylthiopropionic acid ethyl ester, isolated from Katsura-uri (Japanese pickling melon, *Cucumis melo* var. Conomon), enhanced differentiation in human colon cancer cells. *Journal of Agricultural and Food Chemistry* 56(9): 2977-2984.

Nogi, Y., Kanno, T., Nakano, T., Fujita, Y., Tabata, C., Fukuoka, K., Gotoh, A. & Nishizaki, T. 2012. AMP converted from intracellularly transported adenosine upregulates p53 expression to induce malignant pleural mesothelioma cell apoptosis. *Cellular Physiology and Biochemistry: International Journal of Experimental Cellular Physiology, Biochemistry, and Pharmacology* 30(1): 61-74.

Pamu, S., Chen, D., Morin, F., Huo, C., Cui, Q., Dou, Q.P. & Chan, T.H. 2012. Inhibitory effect of bortezomib on human multiple myeloma cells when combined with epigallocatechin-gallate (EGCG) analogs. *MedChemComm* 3(2): 229-232.

Park, J., Kim, H., Kim, S., Lee, D., Kim, M-S. & Shin, D.H. 2018. Crystal structure of D-glycero-B-D-manno-heptose-1-phosphate adenylyltransferase from *Burkholderia pseudomallei*. *Proteins* 86(1): 124-131.

Patel, V., Massenburg, D., Vujicic, S., Feng, L., Tang, M., Litbarg, N., Antoni, A., Rauch, J. & Levine, J. 2015. Apoptotic cells activate amp-activated protein kinase (AMPK) and inhibit epithelial cell growth without change in intracellular energy stores. *Journal of Biological Chemistry* 290(37): 22352-22369.

Rakoczy, K., Kaczor, J., Sołtyk, A., Szymańska, N., Stecko, J., Sleziak, J., Kulbacka, J. & Baczyńska, D. 2023. Application of luteolin in neoplasms and nonneoplastic diseases. *International Journal of Molecular Sciences* 24(21): 15995.

Terkar, A. & Borde, M. 2021. Chapter 6 - Endophytic fungi: Novel source of bioactive fungal metabolites. In *New and Future Developments in Microbial Biotechnology and Bioengineering,* edited by Singh, J. & Gehlot, P. Elsevier. pp. 95-105.

Tomm, H.A., Ucciferri, L. & Ross, A.C. 2019. Advances in microbial culturing conditions to activate silent biosynthetic gene clusters for novel metabolite production. *Journal of Industrial Microbiology and Biotechnology* 46(9-10): 1381-1400.

Vasundhara, M., Reddy, M.S. & Kumar, A. 2019. Chapter 18 - Secondary metabolites from endophytic fungi and their biological activities. In *New and Future Developments in Microbial Biotechnology and Bioengineering,* edited by Gupta, V.K. & Pandey, A. Elsevier. pp. 237-258.

Virtanen, S.S., Kukkonen-Macchi, A., Vainio, M., Elima, K., Härkönen, P.L., Jalkanen, S. & Yegutkin, G.G. 2014. Adenosine inhibits tumor cell invasion via receptor-independent mechanisms. *Molecular Cancer Research: MCR* 12(12): 1863-1874.

Yu, W., Pei, R., Zhou, J., Zeng, B., Tu, Y. & He, B. 2023. Molecular regulation of fungal secondary metabolism. *World Journal of Microbiology and Biotechnology* 39(8): 204.

Zhang, S., Li, Z., Wu, X., Huang, Q., Shen, H-M. & Ong, C-N. 2006. Methyl-3-indolylacetate inhibits cancer cell invasion by targeting the MEK1/2-ERK1/2 signaling pathway. *Molecular Cancer Therapeutics* 5(12): 3285-3293.

*Corresponding author; email: zjhe3@gzu.edu.cn