AaLaeA targets AaFla1 to mediate the production of antitumor compound in *Alternaria alstroemeria*

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Abstract

Endophytic fungi are an important source of novel antitumour substances. Previously, we isolated an endophytic fungus, *Alternaria alstroemeria*, from the medicinal plant *Artemisia artemisia*, whose crude extracts strongly inhibited A549 tumour cells. We obtained a transformant, namely *AaLaeA*^{OE26}, which completely loses its antitumour activity due to overexpression of the global regulator AaLaeA. Re-sequencing analysis of the genome revealed that the insertion site was in the non-coding region and did not destroy any other genes. Metabolomics analysis revealed that the level of secondary antitumour metabolic substances was significantly lower in *AaLaeA*^{OE26} compared to the wild strain, in particular flavonoids were more downregulated according to the metabolomics analysis. A further comparative transcriptome analysis revealed that a gene encoding FAD - binding domain protein (Fla1) was significantly downregulated. On the other hand, overexpression of AaFla1 led to significant enhancement of antitumor activity against A549 with a 7-fold higher inhibition ratio than the wild strain. At the same time, we also found a significant increase in the accumulation of antitumour metabolites including quercetin, gitogenin, rhodioloside, liensinin, ginsenoside Rg2 and cinobufagin. Our data suggest that the global regulator AaLaeA negatively affects the production of antitumour compounds via controlling the transcription of AaFla1 in endophytic *Alternaria alstroemeria*.

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Abstract

Endophytic fungi are an important source of novel antitumour substances. Previously, we isolated an endophytic fungus, *Alternaria alstroemeria* , from the medicinal plant *Artemisia artemisia* , whose crude extracts strongly inhibited A549 tumour cells. We obtained a transformant, namely *AaLaeA* ^{OE26}, which completely loses its antitumour activity due to overexpression of the global regulator AaLaeA. Re-sequencing analysis of the genome revealed that the insertion site was in the non-coding region and did not destroy any other genes. Metabolomics analysis revealed that the level of secondary antitumour metabolic substances was significantly lower in*AaLaeA* ^{OE26} compared to the wild strain, in particular flavonoids were more downregulated according to the metabolomics analysis. A further comparative transcriptome analysis revealed that a gene encoding FAD -binding domain protein (Fla1) was significantly downregulated. On the other hand, overexpression of AaFla1 led to significant enhancement of antitumor activity against A549 with a 7-fold higher inhibition ratio than the wild strain. At the same time, we also found a significant increase in the accumulation of antitumour metabolites including quercetin, gitogenin, rhodioloside, liensinin, ginsenoside Rg2 and cinobufagin. Our data suggest that the global regulator AaLaeA negatively affects the production of antitumour compounds via controlling the transcription of AaFla1 in endophytic*Alternaria alstroemeria* .

Keywords: *Alternaria alstroemeria* , global regulator AaLaeA, FAD-binding domain protein, Antitumor substances, Metabolomics

Introduction

As one of the leading causes of death worldwide, cancer poses a serious threat to human health, and the number of cancer cases is increasing every year [1]. Although we have a variety of means to treat tumours, including surgery, radiation and chemotherapy, all these treatment approaches have certain limitations. Therefore, there is a great need to find new strategies to fight cancer and discover new anti-cancer drugs. Many natural products from fungi have pronounced antitumour activity [2]. Therefore, the search for new structures and active natural products from microorganisms has become an important research direction [3] and has attracted much attention. For example, actinomycin D, daunorubicin, epirubicin, rapamycin and gelanamycin are all microbial derivatives that are used clinically as anticancer drugs [4]. The endophytic fungi as microbial biota from certain niches have also been shown to be able to produce lead compounds and drug candidates for cancer. For example, the crude extract of the ethyl acetate layer of the endophytic fungus isolated from Annona muricata L. was found to have a potent inhibitory effect on MCF -7 cells [5]. Glyconic acid isolated from the endophytic fungus Bionectria sp. showed good cytotoxic activity against the cancer cell line A2780 [6]. Trichodermin terpenoids from Nalanthamala psidii were found to inhibit tumour growth [7]. A p-benzophenone compound cytorhizine isolated from Cytospora rhizophorae showed some inhibitory effect on tumour cells MCF -7, HePG-2, NCI-H460 and SF -268 [8].

With its abundant secondary metabolites and their antitumor activity, fungi in *Alternaria* genus have also attracted the attention of researchers. For example, the secondary metabolites of the fungus*Alternaria alternarta* Y-4-2 isolated from the leaves of*Cephalotaxus oliveri* showed significant antitumor activity against a variety of tumor cell lines including A549, NCI-H460, HL60, NCI-H929 and RPMI8226, including one new dimeric xanthone compound [9]. Two cyclopentaisochromenone enantiomers isolated from*Alternaria* sp. TNXY-P-1 showed antitumor activity against HL-60 cell line [10]. Dibenzo-α-pyrones isolated from *Alternaria*sp. Samif01 possess antibacterial and antioxidant activities [11].

Under laboratory conditions, mostly biosynthetic gene clusters synthesizing secondary metabolites are silenced [12], and low levels of gene expression or expression silencing of secondary metabolic gene clusters affect the production of the corresponding metabolites. LaeA is involved in the biosynthesis of a variety of fungal secondary metabolites [13, 14]. In *Penicillium chrysogenum* , overexpression of LaeA increased penicillin production [15]. DNA microarray analysis of polyketide synthase genes in LaeA deletion mutant strains of *Fusarium verticillioides* indicated that the gene expression of BIK1, FUM1, FUB1, and fusarin was significantly reduced [16]. CglaeA overexpression in *Chaetomium globosum*resulted in an increment of chaetoglobos in A production [17]. These suggest that LaeA could involve in regulating the synthesis of secondary metabolites and its overexpression might increase secondary metabolites including new compounds as well as bio-active substances.

FAD-binding domain protein (Fla1) belongs to the FAD-binding domain gene superfamily and is a flavoenzyme that is widely found in nature [18]; with its ability to catalyze a variety of biochemical reactions, it has been widely used as a biocatalyst for multifarious reactions [19, 20]. About 1% of eukaryotic and prokaryotic proteins are expected to encode flavin adenine dinucleotide (FAD) binding domains [21].

An endophytic fungus viz *Alternaria alstroemeria* was isolated from the medicinal plant *Artemisia annua* early in this study, and its crude extract showed a strong inhibitory effect on A549. Overexpression of the secondary metabolic global regulator AaLaeA discovered a transformant *AaLaeA* ^{OE26} with complete loss of antitumor activity. Via further analyses of the metabolome and transcriptome of mutant strain *AaLaeA* ^{OE26}, we found that the key gene AaFla1 was overexpressed and affecting antitumor activity, meanwhile, the production of antitumor active substance was regulated by the FAD-binding protein in *Alternaria alstroemeria* .

Materials and methods

Identification of AaFla1

The endophytic fungus ZZ-HY-03-02 was identified as *Alternaria alstroemeria* by our group in the early stage, The gene encoding the Fla1 protein of *A. alternata* was used as a template to amplify its ORF frame with Fla1-F and Fla1-R and to identify AaFla1.

Construction of genetic transformation system

A *Fla1* overexpression vector was constructed by double digestion (Xba1, HindIII) strategy. Fla1-F and Fla1-R were used as primers to amplify the *Fla1* gene using *A. alternata* genomic DNA as the template. PtrpC-F and PtrpC-R were used to amplify the promoter PtrpC with the plasmid PtrpC as the template, and the hyg resistance element was amplified with the pK2hyg vector plasmid as the template (Table S1). The two fragments were fused by the overlapping PCR method, and the fusion fragment was ligated into the pK2hyg vector and*electroporated* into *Agrobacterium* . The pK2hyg-PtrpC::Fla1 was transformed into *A. alternata* via*Agrobacterium* -mediated genetic transformation [22].

2.3 Quantitative real-time PCR

The total DNA was extracted from *A. alternata* (WT) and*AaFla1* ^{OE} using a fungal RNA extraction kit (CWBIO, Jiangsu, China). After extraction, the total RNA quality and concentration were measured and DNA was removed, and reverse transcribed into cDNA with reference to the GenStar kit (Genstar, Bei-jing, China). Using a two-step amplification, the quantitative real-time PCR (qPCR) method was pre-denatured at 95°C for 5 min, denatured at 95°C for 30 s, denatured at 56°C for 30 s, and extended at 72°C for 45 s, 40 cycles. The β-Actin gene was used as an internal reference gene (Table S1). After completion of the reaction, the expression was calculated according to the 2^{-T} method to analyze the transcript levels of the genes. All experiments were repeated three times.

2.4 Preparation of strain crude extract

The concentrations of conidia were adjusted to 1×10^6, and then 4 mL of each was added to 500 mL of liquid Sabourand medium. Then, fermentation was carried out by cultivation at 180 rpm on a shaker at 28 °C for 7 days. After fermentation, fluids were extracted using ethyl acetate (proportion 1:1) three times, while the mycelia were stored at -80 °C after quick freezing with liquid nitrogen. The extracts were dried in a rotary evaporator and dissolved in 1 mL of dimethyl sulfoxide. The crude extracts were used in subsequent cellular assays and analyzed the antitumor activity substances with the metabolomic assay. The metabolomic assay was entrusted to BaySpectrum Biologicals (https://www.bioprofile.cn).

2.5 Antitumor activity test

The lung cancer A549 cells in the logarithmic growth phase state were taken and adjusted to 1×10^5 cells/mL, and 100 μL cells per well were added into a 96-well plate. At the same time, the blank group and the normal cell control group were cultured overnight at 37 , 5% CO_2. The positive drug (adriamycin) and

the crude extract were dissolved and filtered in dimethylsulfoxide (DMSO). Then, the cells were treated with different concentration gradients of adriamycin and the crude extract for 48 h, respectively. MTT (20 μL) was added to each well and incubated at 37 for 4 h. After adding 150 μL DMSO, the absorbance OD_{490} was determined by a microplate reader (BIOBRI, Chengdu, China).

2.6 Detection of apoptosis via annexin V–FITC/PI method

A549 cells in the logarithmic growth phase and in good growth condition were digested by trypsin, and the reaction was terminated by adding RPMI1640 fresh medium containing serum and centrifuged to make cell suspension. Inoculated in 6-well plates at 1×10^6cell/mL, 2 ml per well, and incubated at 37 for 24 h in a 5% CO_2 incubator; When the cells grew to about 80%, they were treated with 300, 150, and 75 μg/mL WT and*AaFla1* ^{OE23} mutant crude extracts for 48 h. The cells were then collected, washed 3 times with 1xPBS, processed by centrifugation, and stained according to Annexin V-FITC/PI kit before being detected by flow cytometry for apoptosis.

2.7 LC-MS/MS analysis

The samples were analyzed in a 4°C autosampler using a SHIMADZU-LC30 ultra-high performance liquid chromatography (UHPLC) system with an ACQUITY UPLC® HSS T3 (2.1×150 mm, 1.8 μm) (Waters, Milford, MA, USA) column. The mobile phase A: 0.1% formic acid aqueous solution, B: acetonitrile; the chromatographic gradient elution procedure was as follows: 0 - 1.5 min, 0.3% B; 1.5 - 2 min, B linearly varied from 0.3% to 0%; 2 - 6 min, B linearly varied from 0% to 48%; 6 - For 10 min, B varied linearly from 48% to 10%; for 10-12 min, B was maintained at 100%; for 12-12.1 min, B varied linearly from 100% to 0%; for 12.1-15 min, B was maintained at 0%.

Each sample was detected in positive (+) and negative (-) ion modes by electrospray ionization (ESI). The samples were separated by UPLC and analyzed by mass spectrometry using a QE Plus mass spectrometer (Thermo Scientific), and ionized using an HESI source with the following ionization conditions: Spray Voltage: 3.8kv (+) and 3.2kv (-); Capillary Temperature: 320 (±); Sheath Gas: 30 (±); Aux Gas: 5 (±); Probe Heater Temp: 350 (±); S-Lens RF Level: 50.

2.8 Transcriptome analysis

The RNA samples were tested for purity, concentration, and integrity. A total RNA amount of 1 μg per sample was used as input material for RNA sample preparation to ensure that qualified samples were used for transcriptome sequencing. Sequencing libraries were generated using the NEBNext®Ultra RNALibrary Prep Kit for Illumina(r) (NEB, USA), adding manufacturer and index codes to the attribute sequences of each sample. The cDNA first strand was synthesized using hexamer primers and reverse transcriptase. Subsequent synthesis of the cDNA second strand was performed using DNA polymerase I and RNase H. Once the 3' ends of the DNA fragments were adenylated, the NEB Next Adapter was ligated and prepared for hybridization. The library fragments were purified using the AMPure XP system (Beckman Coulter, Beverly, USA) to select cDNA fragments of 240 bp in length. The fragment size was then picked with 3 μL of USER enzyme (NEB, USA), and the cDNA was ligated at 37 °C for 15 min and at 95 °C for 5 min for PCR. PCR was then performed with Phusion high-fidelity DNA polymerase, universal PCR primers, and Index (X) primers. finally, the PCR products were purified and the sequences were analyzed on an Agilent Bioanalyzer the library quality of the obtained sequences was evaluated on the Agilent Bioanalyzer 2100 system.

2.9 Statistical analysis

Statistical analysis was performed with SPSS 20.0 software. All of the data were expressed as the mean ± SEM. $P < 0.05$ was considered statistically significant. RT-PCR and cell experiments results were analyzed using Student's t test.

Results

3.1 Loss of antitumor activity in an *AaLaeA*^{OE26}

To clarify the status of A549 cells, the assay was performed with adriamycin as the positive drug. The results found the IC_{50} of adriamycin against A549 was 2.63 μg/mL, which is consistent with the literature [23]. It showed that the A549 cell line was in normal condition and could be used for the experiment (Fig. 1A). Overexpression of the global regulator AaLaeA in *Alternaria alstroemeria* led to two contrasting antitumor activities in overexpressed transformants. Among them, the transformant *AaLaeA* ^{OE29} showed increased antitumor activity [24], while *AaLaeA* ^{OE26} completely lost its antitumor activity (Fig. 1B). The crude extract of the wild-type strain of *A. alstroemeria* showed significant inhibition of A549 cells, with the relative inhibition rate in a concentration-dependent manner. At a concentration of 300 μg/mL, the inhibition rate reached 33.43 % and the IC_{50} value was 328 μg/mL. This indicated that the crude extract of the wild-type strain had a significant inhibitory effect on A549 (Fig. 1B). The insertion site of *AaLaeA* ^{OE26} was analyzed via genome resequencing, and the results showed that the insertion site of *AaLaeA* ^{OE26} was in the non-coding region. Therefore, we suggest that the insertion of *AaLaeA* did not destruct other genes (Fig. S1). The data showing the loss of antitumor activity of *AaLaeA* ^{OE26} was caused by the expression of AaLaeA, and there may be a quantitative-effect relationship between AaLaeA expression and antitumor activity.

3.2 AaLaeA is involved in the metabolism of antitumor substances and regulates the expression of metabolism-related genes

To investigate the material basis of the loss of antitumor activity of *AaLaeA* ^{OE26}, LC/MS nontargeted metabolomics analysis of the endophytic fungus *A. alstroemeria* WT and *AaLaeA* ^{OE26} was performed. Principal component analysis (PCA) showed that there was significant separation between the WT and *AaLaeA* ^{OE26} samples, with PC1 and PC2 accounting for 95.14% and 4.44% of the total variation, respectively (Fig. 2A). Hierarchical cluster analysis and Plots from partial least squares discriminant analyses (PLS-DA) likewise showed significant separation of WT and *AaLaeA* ^{OE26} metabolites (Fig.S2); Further analysis of the differing substances revealed noteworthy metabolic differences between the two groups of samples (Fig. 2B). Screening for metabolites($\log_2$ FC > 0.5、 P value < 0.05 and VIP > 1) with significant differences revealed that the flavonoid epicatechin gallate [25] was the most significantly down-regulated of the top 10 live compounds (Table 1) in terms of anti-tumour compounds; Phenolic and indole derivatives were also included in the down-regulated antitumour activators (Fig. 2C). Through KEGG pathway enrichment, it was found that differential metabolites were enriched into 20 metabolic pathways, including fatty acid synthesis, amino acid synthesis and indole alkaloid synthesis (Fig. 2D). The above results suggest that significant down-regulation of several major classes of antitumour compounds in the *AaLaeA* ^{OE26} strain might be responsible for the loss of antitumour activity in *AaLaeA* ^{OE26}.

To clarify the molecular basis of the loss of antitumor activity of *AaLaeA* ^{OE26}, the transcriptome patterns of WT and *AaLaeA* ^{OE26} were analyzed comparatively at the same time points as the above metabolome sampling. Analysis of the depth of sequencing and the clean reads obtained showed that the samples all reached 5.90 Gb of Clean Data and over 19,755,288 Clean reads (Table 2), all meeting transcriptome quality standards. A total of 3795 differential genes ($|\log2\text{FoldChange}| > 1$, FDR < 0.05) were detected, of which 1893 were up-regulated and 1902 were down-regulated (Fig. 3A). GO functional analysis showed that the differential genes were mainly involved in substance metabolic processes, catalytic enzyme activity and binding activity (Fig. 3B). Metabolic pathway clustering analysis showed that diverse genes were mainly involved in four pathways: metabolism, genetic information processing, cellular processing and environmental information processing (Fig. 3C). This suggests that AaLaeA is involved in the metabolism of *A. alstraoemeria* and may be involved in the regulation of catalase genes in the metabolic process.

3.3 AaFla1 mediates the anti-tumour activity of *A. alstroemeria*

To further investigate the molecular mechanisms underlying the loss of antitumour activity of *AaLaeA* ^{OE26}, the results of the metabolomic analysis were combined and found that flavonoids (epicatechin gallate) might contribute prominently to the antitumor activity of *AaLaeA* ^{OE26}. Therefore, we hypothesized that AaLaeA primarily regulated flavonoid synthesis in *AaLaeA* ^{OE26}. And then, we did an in-depth analysis of metabolomic and transcriptomic data, and a total of 86 genes involved in flavonoid metabolism and antitu-

mour related were obtained (Table S2). The gene number 3698 encoding FAD-binding domain protein (Fla1) (Fig.4A) was mostly significantly down-regulated in *AaLaeA* ^{OE26}. The expression of this gene in *AaLaeA* ^{OE26} was verified using qRT-PCR and found to be consistent with the results of transcriptome analysis (Fig. 4B). To confirm whether Fla1 mediates the antitumour activity of the fungus, *Fla1* was overexpressed in *A. alstroemeria* wild type (Fig. S3). It was found that overexpression of Fla1 led to a significant increment in the antitumour activity of the strain. The IC_{50} of *AaFla1* ^{OE23} against A549 was 368 μg/mL, significantly lower than that of WT at 445 μg/mL (Fig. 4C); The rate of apoptosis was also significantly increased (Fig. S4), about 4.72-fold higher than WT (Fig. 4D). Those suggest that Fla1 mediates the antitumour activity of *A. alstroemeria* .

3.4 FAD-binding domain proteins mediate the production of antitumour active substances

To investigate the material basis of AaFla1 mediated antitumour activity, differential metabolites between WT and *AaFla1* ^{OE23} strains were analyzed using LC/MS non-targeted metabolomics. Principal component analysis (PCA) showed that there was a significant separation between the WT and *AaFla1* ^{OE23} samples, with PC1 and PC2 accounting for 48.34% and 14.72% of the total variation, respectively (Fig. 5A). Hierarchical cluster analysis showed that the 2 sets of samples shared two branches, with both the *AaFla1* ^{OE23} and WT repeats forming a separate branch, indicating significant metabolic differences between them (Fig. S5A); Analysis of metabolite differences between WT and *AaFla1* ^{OE23} from partial least squares discriminant analyses (PLS-DA) also showed the difference (Fig. S5B and C). The results of the above analyses showed that there were significant differences in secondary metabolites between WT and *AaFla1* ^{OE23} (Fig. 5B).

Screening of differential metabolites ($Log_2FC > 0.5$, P value < 0.05, and VIP > 1) for the top 10 up-regulated differential metabolites (Table 3) and Cluster analysis of the differential substances (Fig.5C) revealed that the differential metabolites were alkaloids, carbohydrates, lipids, organic acids, peptides, PK polyketides, steroids, and terpenoids. Further enrichment by the KEGG pathway revealed that the metabolic pathways involved in the differential substances included flavonoids, cofactors, unsaturated fatty acids, various secondary metabolites, and amino acid metabolism as biosynthetic pathways were enriched (Fig. 5D). Some compounds with anti-tumor activity were found to be significantly up-regulated (Table 3). Quercetin [26], Strychnopentamine [27], Gitogenin [28], Rhodioloside [29], Liensinine [30], L-Selenomethionine [31], Compactin [32], Tonantzitlolone B [33], Ginsenoside Rg2 [34], Campesterol [35], Pristimerin derivative [36], Cinobufagin [37] and Anisomycin [38] have been shown to have antitumour activity. Among them, quercetin, a flavonoid, was the most significantly up-regulated.

To investigate the effect of overexpression of AaFla1 on antitumour active substances, the top ten differential metabolites of WT and *AaFla1* ^{OE23} metabolic groups (Table 2) and WT and *AaLaeA* ^{OE26} metabolic groups (Table 3) were compared (Table 4). Overexpression of LaeA resulted in down-regulation of Fla1 expression and reduced antitumour activity in *AaLaeA* ^{OE26}, with the most pronounced down-regulation of antitumour flavonoids in the analyzed metabolome. Overexpression of Fla1 elevated the antitumour activity of *AaFla1* ^{OE23}, and the most up-regulated antitumour compounds in the *AaFla1* ^{OE23}metabolome were flavonoids.

Taken together, the results indicate that the global regulator AaLaeA negatively regulates the AaFla1 gene, that AaFla1 regulates the production of antitumour active substances in the strain, and that the *A. alstroemeria* FAD-binding domain protein regulates the production of antitumour active substances.

4. Discussion

Currently, anti-neoplastic drugs are more or less deficient in terms of weak targeting, toxic side effects, and drug resistance [39]. The search for novel antitumour drugs with low side effects and high targeting is one of the important topics of research in the field of medicinal chemistry. Plant endophytic fungi are an important source of novel antitumour compounds due to their unique ecological niche, which harbours unique and novel compound structures. A strain of *Alternaria alstroemeria* was discovered and isolated by our group, and its crude extract showed a strong inhibitory effect on A549 [24]. To investigate the molecular basis of the antitumour activity of this strain, the global regulator of secondary metabolism viz AaLaeA was

overexpressed. It was found that *AaLaeA* ^{OE29}crude extract inhibited A549 significantly more than WT and increased the total apoptosis rate by 1.56-fold and AaLaeA mediated the antitumour activity of the crude extract [24]. The function of LaeA has been reported to be conserved in previous studies and is involved in the biosynthesis of a variety of fungal secondary metabolites [13]. Overexpression of LaeA or its homologs in *Penicillium chrysogenum*[15], *Chaetomium globosum* [17], and *Aspergillus nidulans* [40] all resulted in a large increment in fungal secondary metabolites; and these reports are consistent with the results of our group's previous studies. Interestingly, when we screened for AaLaeA overexpression strains, we found a transformant numbered 26 with 10-fold higher expression of AaLaeA than WT, but with a significant loss of antitumour activity. To clarify whether the insertion was caused by a disruption of the relevant gene at the insertion site, the transformant was subjected to genome re-sequencing, which revealed that no coding gene was present upstream or downstream of the insertion site and that the insertion was a nonsense insertion. It is hypothesized that the AaLaeA-mediated loss of antitumour activity is not directly correlated with the insertion site, but may be quantitatively related to the expression of AaLaeA itself. Metabolomic analysis revealed that*AaLaeA* ^{OE26} contained significantly lower levels of secondary metabolites of antitumour activity compared to *A. alstroemeria* wild type. These differential metabolites were carboxylic acids and their derivatives, fatty acids, phospholipid glycerols, benzene and its substituents, flavonoids, phenols, indole derivatives, and other intermediate derivatives which were reported to have the potential for antitumour activity. In particular, the flavonoids were more significantly down-regulated, with the most significant down-regulation and the largest fold difference being in the flavonoid epicatechin gallate. In combination with comparative transcriptome analyses, a significant down-regulation of the gene encoding the FAD-binding domain-containing protein (Fla1) was found; Fla1 belongs to the FAD-binding domain superfamily and is a flavoenzyme that is widely found in nature [18]; It is capable to catalyze a variety of biochemical reactions and has been widely used as a biocatalyst for a variety of reactions, and many biochemical processes take advantage of the versatility of flavoenzymes and extremely such as flavin cofactors [19, 20]. It is hypothesized that AaFla1 may be negatively regulated by AaLaeA, thereby affecting the synthesis of the antitumour compounds in *A. alstroermeria* . To further verify the relationship between AaLaeA and AaFla1 genes, the transcript levels of Fla1 in wild-type and*AaLaeA* ^{OE26} strains were analyzed by qRT-PCR. The results showed that overexpression of AaLaeA resulted in a significant down-regulation of Fla1 transcription. In contrast, overexpression of Fla1 caused a significant increment in the antitumour activity of the strain, with a reduction in IC_{50} from 445 μg/mL to 368 μg/mL and a significant 7-fold increment in apoptosis. Antitumor-related compounds have been reported to accumulate in Fla1 overexpressing transformants, for example, Quercetin [26], Strychnopentamine [27], Gitogenin [28], Rhodioloside [29], Liensinine [30], L-Selenomethionine [31], Compactin [32], Tonantzitlolone B [33], Ginsenoside Rg2 [34], Campesterol [35], Pristimerin Derivative [36], Cinobufagin [37] and Anisomycin [38], with the flavonoid Quercetin being the most significantly up-regulated. This result is in contrast to the significant reduction in flavonoid content of*AaLaeA* ^{OE26}, which also confirms in terms of compound accumulation that AaFla1 is negatively regulated by AaLaeA, and therefore affecting the antitumour activity of the strain.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Abbreviations

Abbreviations	Full name
MTT	3 - (4, 5- dimethylthiazol - 2 - yl) -2, 5- diphenyltetrazolium bromide
ADM	Adriamycin
FC	fold change difference multiple
VIP	variable projection significance assessment
DEGs	differentially expressed genes

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