科技部補助專題研究計畫成果報告
期末報告

利用異源系統表現紅麴菌之二次代謝產物

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中 文 摘 要 ： 真菌的非核糖體胜肽合成酶(nonribosomal peptide synthetases, NRPSs)屬於多功能蛋白質複合體，其二次代謝產物廣泛被應用在醫療製藥界，這些藥用價值歸因於二次代謝產物特殊功能，例如著名的紅麴菌素K(monacolin K)是由紅麴菌(Monascus pilosus)之聚酮合成酶(polyketide synthases, PKSs)負責生合成，紅麴菌素K是一種膽固醇合成抑制劑，能調節人體內的膽固醇。另外有研究顯示，麴菌所產生的黃酮酚(flavonoid)可增加天然抗氧化能力。已知紅麴菌(Monascus)屬於子囊菌門(Ascomycota)，其中的Monascus ruber及Monascus purpureus已完成基因組定序，目前依紅麴菌進行基因探勘的結果顯示，所鑑定出的二次代謝產物並不多，意味著許多生合成基因的產物有待進一步鑑定。小巢狀麴菌(Aspergillus nidulans)屬於子囊菌門之絲狀真菌，因其生長快速且易於培養、架構及遺傳背景簡單且易於分析等優勢，使其成為真核細胞生物學上重要的研究工具。本計畫利用小巢狀麴菌為異源表現(heterologous expression)系統，表現M. ruber的非核糖體胜肽合成酶基因(包含PID 398062, PID3 83194, PID 391237, PID 500739, PID 441704 and PID 499644等)，並分別偵測其蛋白所負責的生合成產物，本研究結果顯示，在透過可調節的啟動子alcA(P)進行轉錄活化後，PID 500739非核糖體胜肽合成酶的異源表達株產生化合物1 (m/z 314 MH) 與化合物2 (m/z 466 MH)，此結果顯示透過異源表達系統確實可針對紅麴菌中二次代謝產物之基因體功能進行研究與解析，除有利於進一步鑑定其結構並深入探討其生合成叢集之產物外，對真菌在研製保健與醫療相關應用上也提供重要資訊。

中 文 關 鍵 詞 ： 非核糖體胜肽合成酶，二次代謝產物，紅麴菌，異源表現

英 文 摘 要 ： Nonribosomal peptide synthetases (NRPSs) are multimodular enzymes that produce a diverse group of natural products. The efficacy as medicines is due to the biological activities of secondary metabolites (SMs). Monacolin K, an antihypercholestrolemia agent, is a secondary metabolite synthesized by PKSs from Monascus. Recent reports showed that the enhanced contents of flavonoids in the Monascus-fermented rice bran (MRB) may contribute to the antioxidant activity in MRB. Monascus is species of mold and belongs to the phylum Ascomycota. The genomes of two species of Monascus, Monascus ruber and Monascus purpureus, which are commonly used for Monascus-fermented rice (MFR), have been sequenced. The number of known secondary metabolites (SM) produced by M. ruber and M. purpureus is much less than the SM genes that have been identified. Our preliminary results in heterologous overexpression system revealed that Monascus polyketide synthase (PKS) genes can be expressed in A. nidulans and produced analyzable products. To further confirm if this is the case in NRPS, we further use A. nidulans as a heterologous overexpress host to express the NRPS genes of M. ruber. Among them, we found that the overexpression of PID 500739 results in the production of

compound 1 (m/z 314 MH) and 2 (m/z 466 MH) using liquid chromatography/mass spectroscopy (LC/MS) analysis. Our data suggested that the identification of the important secondary metabolites from M. ruber will provide a basis for the clues of NRPs biosynthetic pathways. This grant will help to unlock the metabolites from the M. ruber and provide useful development resources for healthcare and medical centers.

英文關鍵詞：nonribosomal peptide synthetase, secondary metabolite, Monascus, heterologous expression

前言

Due to their medicinal properties, fungi have been used in Traditional Chinese Medicine (TCM) broadly such as *Monascus*, *Ganoderma*, and *Cordyceps* (2,10,11). Most of these fungi are rare in the wild and grow only in certain regions on specific substrates. The therapeutic effects of TCM are mainly due to their production of bioactive secondary metabolites (SMs) and many important SMs have been isolated and characterized from TCM fungi (8,9,10). Because of the medicinal significance of TCM fungi, genome sequencing efforts have been initiated in the past few years (2,13). More recently, large sequencing centers such as the Joint Genome Institute (JGI) of the Department of Energy have embarked on large scale sequencing efforts such as the 1000 Fungal Genomes Project (6), which includes TCM fungi. The genomic sequencing data has also shown that these gene clusters generally more than the number of compounds known to be produced (1,5,7). Thus, many SM biosynthetic clusters are cryptic and they are not expressed at significant levels under normal culture conditions.

Monascus species are known to possess a variety of medicinally beneficial activities. *M. ruber* (NRRL 1597, ATCC13692) have been sequenced and annotated by JGI. The genomes of *M. ruber* were analyzed using homology and domain analysis and identified core natural product biosynthesis genes such as polyketide synthase (PKS), nonribosomal peptide synthetase (NRPS), and hybrid PKS/NRPS genes. To facilitate the characterization of cryptic secondary metabolomes, in this proposed grant period, we conducted heterologous expression approach to expression cryptic SM genes of *M. ruber* in the model fungus *Aspergillus nidulans*.

研究目的

There are many good reasons to identify the products of cryptic SM clusters of TCM fungi. First, these cryptic clusters may produce compounds that are important for the medicinal activity of TCM fungi. Second, some normally silent SM gene clusters may produce toxic compounds when expressed. Identifying toxins that the organism has the capacity to produce will help us understand potential hazards and thus avoid them. Third, TCM cryptic SM pathways may produce compounds which are useful for other than medical purposes. Lastly, the final compounds produced by the fungi are likely optimized by evolution to benefit the fungus. Our long-term goal is to elucidate the secondary metabolomes of TCM fungi as fully as possible and we believe our work with *Monascus* will not only produce interesting and useful data but will also help refine tools required to leverage the emerging data on the genomes of TCM fungi to understand the mechanisms of action of TCM fungal products and to produce medically useful compounds.

文獻探討

The impact of the JGI's efforts is tremendous because all of the genome information is publicly available and the reference strains are freely available to the research community. *M. ruber* and *M. purpureus* are two of the three species commonly used to produce *Monascus*-fermented rice [(MFR) aka Hung-Chu, Hong Zu, Ang-kak, Ankak rice, red mold rice, Beni-Koji and red yeast rice (an incorrect term since *Monascus* is not a yeast)]. *Monascus* species are known to possess a variety of medicinally beneficial activities. The most prominent is efficacy in cholesterol management. In large part this is due to the production of monacolins,

which were the first statin anti-cholesterol agents to be discovered. As we know, monacolin K was first isolated from *M. ruber* (4). Monacolin K inhibits HMG-CoA reductase, the rate limiting factor in cholesterol biosynthesis. Interestingly, however, if one compares the effects of MFR with an equivalent amount of purified monacolin K, MFR is more potent in reducing cholesterol, LDL cholesterol and triglycerides. This indicates that components of MFR other than monacolin K have positive effects on hypercholesterolemia.

研究方法

To generate transforming fragments by fusion PCR

We first design primers to amplify the *NRPS* genes in *M. ruber* (NRRL 1597, ATCC13692) by polymerase chain reaction (PCR) method. The *yA* gene 5' flanking region-linked with the *pyrG* marker and the *alcA* promoter, and the *yA* gene 3' flanking region were amplified from the genomic DNA of *A. nidulans* strain. Each *NRPS* gene was fused to the *yA* gene 5' region flanking-linked the *pyrG* marker with the *alcA* promoter and the *yA* gene 3' flanking region. These constructs were generated by fusion PCR to replace the native promoters of the *NRPS* genes with a regulatable *alcA* promoter (3, 12).

A. nidulans protoplasting and transformation

The protoplast production and transformation were carried out as described (12). The transformants with the correct insertion were further verified with diagnostic PCR with *yA* P1 primer and (gene) AR primer. In each case, at least three transformants carrying the correct promoter replacement were used for further study.

Fermentation

For fermentation, 3.0×10^7 spores of *A. nidulans* were grown in 125-ml flasks containing 30 ml liquid LMM medium (15 g/l lactose, 6 g/l $NaNO_3$, 0.52 g/l KCl, 0.52 g/l $MgSO_4\cdot7H_2O$, 1.52 g/l KH_2PO_4, and 1 ml/l trace elements) supplemented with uracil (1 mg/ml), uridine (10 mM), riboflavin (2.5 mg/l), or pyridoxine (0.5 mg/l) when necessary at 37 °C with shaking at 200 rpm. For alcA promoter induction, cyclopentanone at a final concentration of 10 mM was added to the medium after 18 h of incubation. Culture medium was collected 48 h after cyclopentanone induction by filtration and extracted with the same volume of ethyl acetate and evaporated *in vacuo*, redissolved in 0.5 ml of 1:4 dimethyl sulfoxide/MeOH, and 10 µl was injected for high performance liquid chromatography–diode-array detection–mass spectrometry (HPLC-DAD-MS) analysis. Conditions for MS included a capillary voltage 5.0 kV, a sheath gas flow rate at 60 arbitrary units, an auxiliary gas flow rate at 10 arbitrary units, and the ion transfer capillary temperature at 350 °C. HPLC-MS was carried out in positive mode using a ThermoFinnigan LCQ Advantage ion trap mass spectrometer with an RP C18 column at a flow rate of 125 µl/min.

結果與討論（含結論與建議）

Our preliminary data indicated that expression the NR-PKS genes of *M. ruber* results in compound production in our heterologous system. It's important to confirm if this is the case in NRPS. First, we analyzed the domain structures of NRPSs in *M. ruber* by using Conserved Domain Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The domain structure of each NRPS were shown in Table 1. Among them, we first focused on the proteins with special architectures including PID 383194, PID 398062, PID 391237, PID 500739, PID 441704 and PID 499644.

Protein ID	Domain struc
383194	C-A-T-C
404613	T-C-A-T-C-A-T-C
398062	A-T-C-A-T-C
439496	A-T-C-A-T-C-A-T-C-A-T-C-T
471310	A-T-C-A-C-A-T-C-A-C-A-T-C
500739	C-A-T-R
499644	A-T-C-A-T-C

Table 1. The domain of NRPSs in *M. ruber*.

Here, we conducted a heterologous expression system that builds on the fusion PCR techniques to express these NRPSs in *A. nidulans*. The transforming fragments was generated and each NRPS gene was placed under control of a regulatable promoter. First, we amplified target genes from genomic DNA of *M. ruber* by PCR, place them under control of *alcA* promoter, *alcA*(p) and insert them into the *yA* locus of *A. nidulans* (Figure 1). The *yA* gene is responsible for synthesis of green conidial pigment. When the *yA* gene was disrupted, the strain produces yellow spores that facilitate the identification of desired clones.

(A)

Primer	Sequence (5' to 3')
yA-P1	TTCTTCCAGCTTCTGCTGCGT
yA-P2	CGACAACCAAGGGAAGTCAA
yA-P3	CGCATTCTAGAGAGAGTGTG
yA-P4	ATTGCGTCCCATCAAATGGG
yA-P5	CAAACTCCTTGACACCGT
yA-P6	GAGTCTGCAGCAAAGGCATTGA

(B)

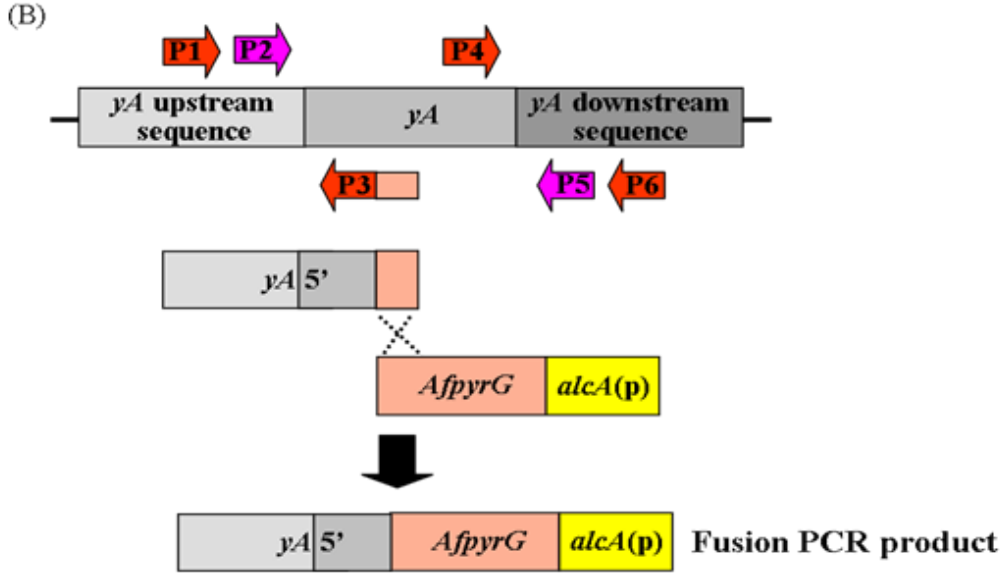

Figure 1. Genetic manipulation of the *yA* gene in *A. nidulans*.

Refactoring these genes into *A. nidulans* would require that these genes be fused to selectable markers, promoters and targeting sequences. By fusion PCR, the target gene fragment is fused to a cassette that consists of 5' flanking region from the *yA* locus of *A. nidulans* as well as a selectable marker (*AfpyrG*) and regulatable promoter *alcA*(p) and 3' flanking region from the *yA* locus (Figure 2). Homologous recombination is extremely accurate and efficient in *A. nidulans nkuAΔ* strains. The transforming fragments integrate by homologous recombination at the *yA* locus. The result is a full length target gene coding region under control of *alcA*(p) integrated at the *yA* locus. The transformant colonies having yellow spores so correct integrates are easily identified when the *yA* gene is deleted. The resulting strains will be verified by diagnostic PCR.

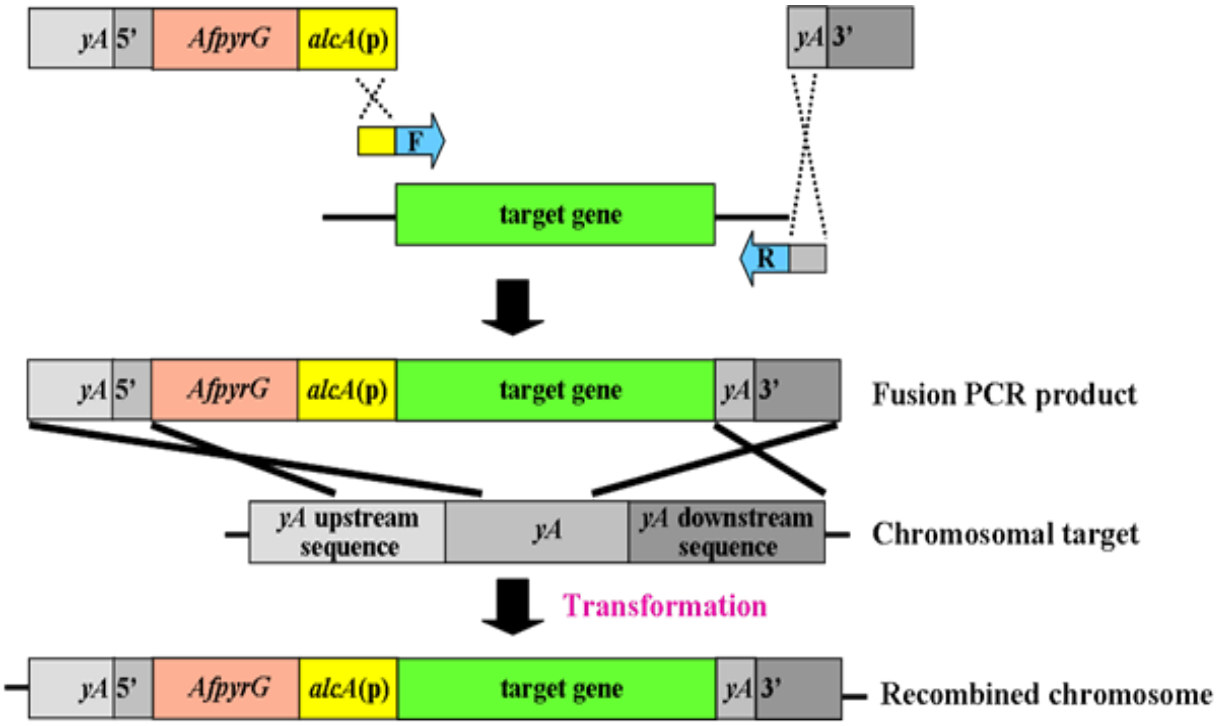

Figure 2. Genetic manipulation streatage for heterologous expression the *M. ruber* genes in *A. nidulans.*

First, target genes from genomic DNA of *M. ruber* were amplified by PCR using specific primers (Table 2). After PCR amplification, the reaction mix were underwent gel electrophoresis and band elution to clean up the target fragments to avoid the unwanted DNA contaminations (Figure 3).

Primer	Sequence (5' to 3')
Mr398062-AF	CCAATCCTATCACCTCGCCTCAAAATGTCCAATCAAAGCACGGC
Mr398062-R	CCCATTTGATGGGACGCAATGGATGAGACAGGACTGACC
Mr500739-AF	CCAATCCTATCACCTCGCCTCAAAATGGGGGTCGAATCAGTCTC
Mr500739-R	CCCATTTGATGGGACGCAATGCTTACTTCTAGCCTCAG
Mr383194-AF	CCAATCCTATCACCTCGCCTCAAAATGAGTGTCATTTCGGGTCC
Mr383194-R	CCCATTTGATGGGACGCAATCAGCTACTACTTCGTGTTG
Mr441704-AF	CCAATCCTATCACCTCGCCTCAAAATGTTAGGGTTGAAGGCGAT
Mr441704-R	CCCATTTGATGGGACGCAATCTTTTGTCGAGCGCAATAG
Mr391237-AF	CCAATCCTATCACCTCGCCTCAAAATGTCGCTACGGGACCACTT
Mr391237-R	CCCATTTGATGGGACGCAATCGACACTTCAGGATCTGTC
Mr499644-AF	CCAATCCTATCACCTCGCCTCAAAATGGGCTCTATACACATCGA
Mr499644-R	CCCATTTGATGGGACGCAATGATACGTATTTCCTGCATC

Table 2. Primer design for PCR amplification of *NRPS* genes of *M. ruber*.

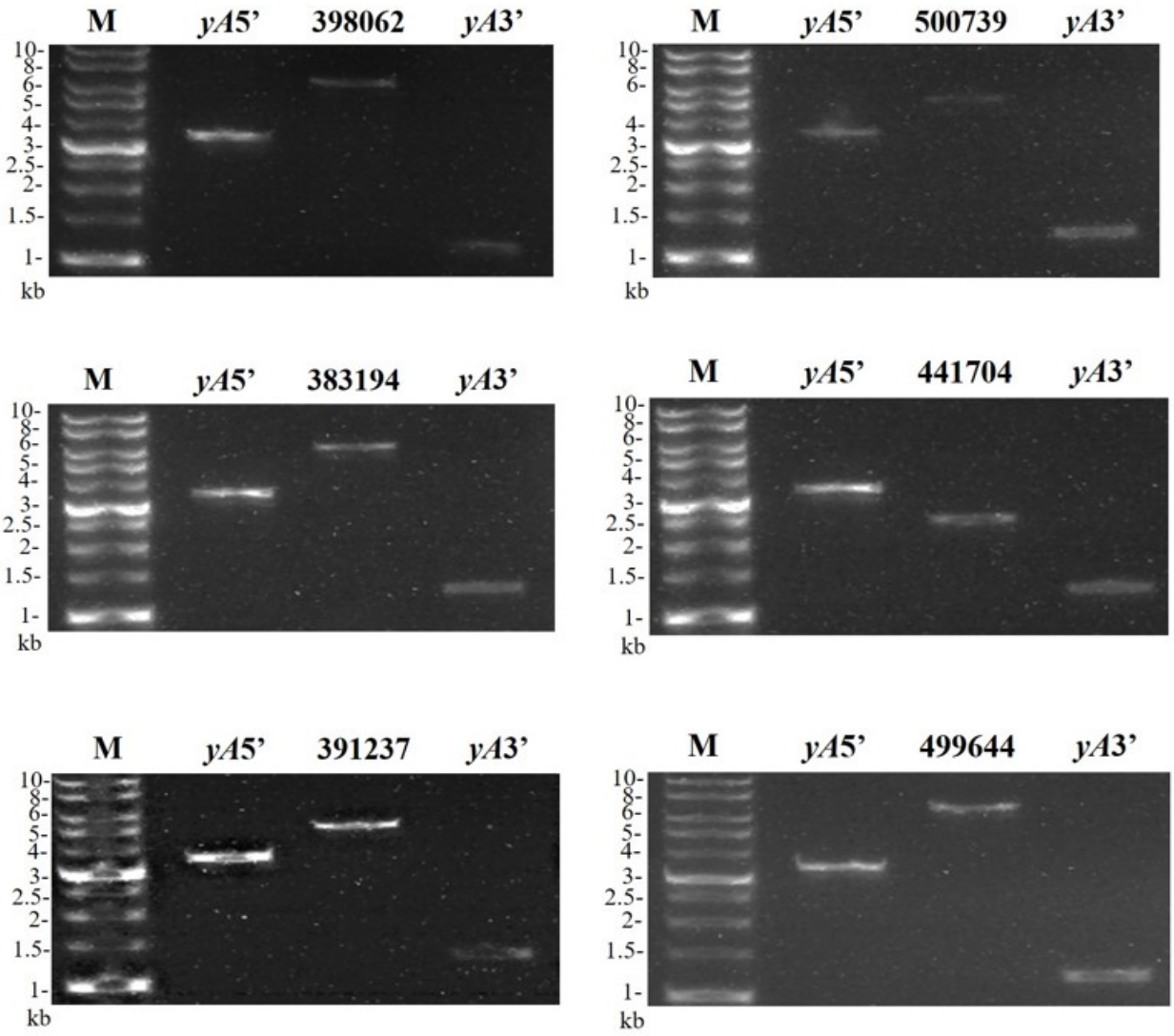

Figure 3. Gel electrophoresis analysis of the *yA5'-AfpyrG-alcA*(p) fragment*, NRPS* genes and *yA 3'* fragment that used for fusion PCR.

Next, the target gene fragments as mentions above (including PID 398062, PID3 83194, PID 391237, PID 500739, PID 441704 and PID 499644) were fused to a *yA* 5'-*AfpyrG-alcA*(p) fragment (consists of 1 kb of 5' flanking DNA from the *yA* locus of *A*. *nidulans*, a selectable marker (*AfpyrG*) and regulatable *alcA* promoter) and 1 kb of 3' flanking DNA from the *yA* locus using fusion PCR methods. The results were underwent agarose gel electrophoresis to verify the transforming DNA size and DNA amount as well (Figure 4).

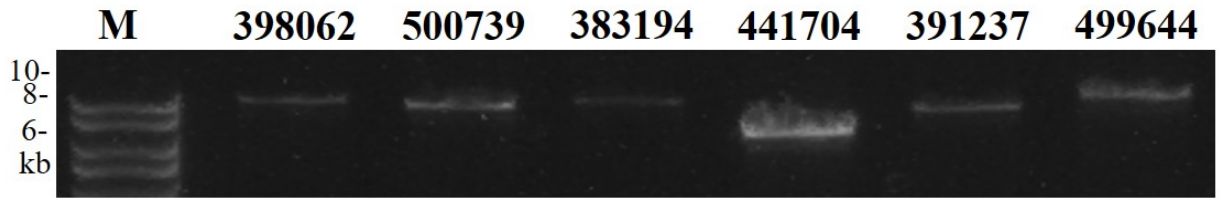

Figure 4. Generation of transforming fragments by fusing the target gene with the *yA* 5'-*AfpyrG-alcA*(p) fragment and *yA* 3' fragment using fusion PCR methods

After transformation, the *yA*-disrupted mutants were easy to be distinguished due to the foreign insert has disrupted the function of *yA* gene and thus produce yellow spore pigments (Figure 5A). We also further confirm the *yA*-disrupted mutants genetically with diagnostic PCR by using *yA* P1 and (gene) AR primers and the desired size of each mutants were indicated as Mr398062: 7068 bp, Mr500739: 6328 bp, Mr383194: 6714 bp (Figure 5B). Next, the metabolite profiles of genetic manipulation strains were monitored using LC/MS analysis. These data showed that the overexpression of PID 500739 results in the production of compounds **1** and **2** in comparison with the *A*. *nidulans* host (Figure 5C).

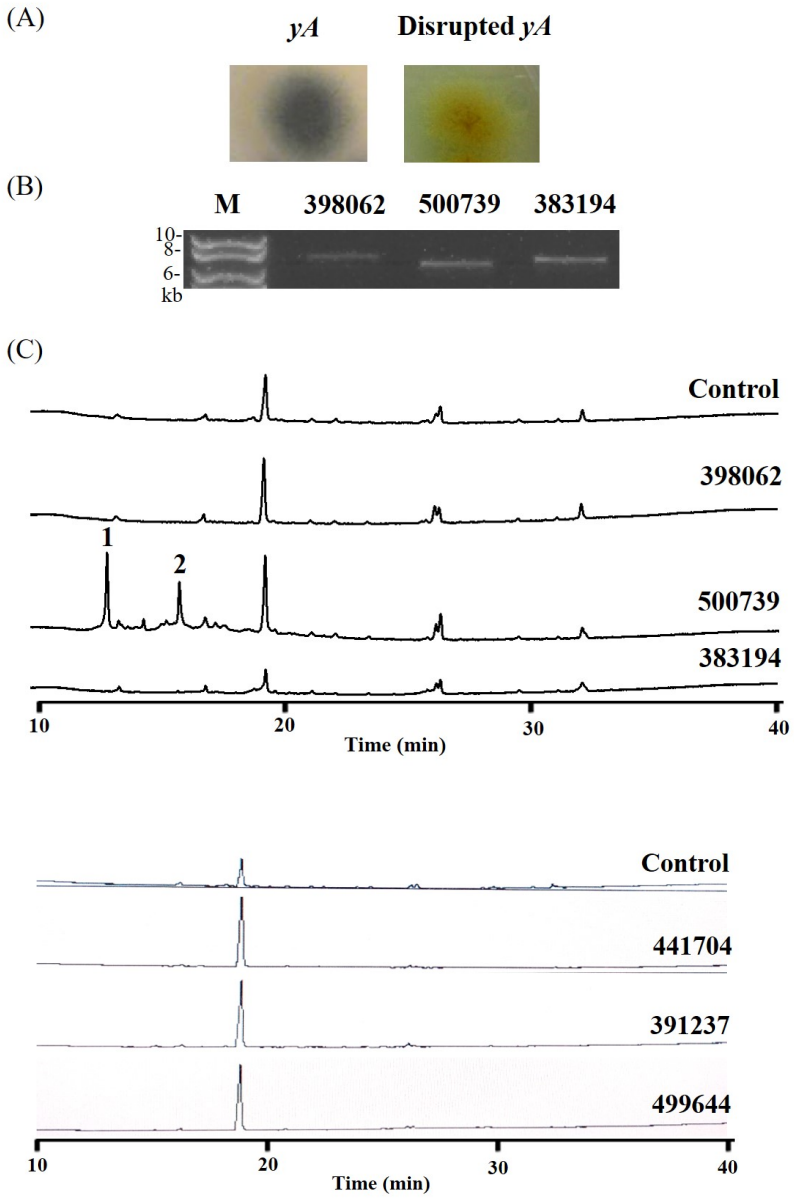

Figure 5. Heterologous overexpression of the *M. ruber NPRS* genes in the *A. nidulans* host. (A)*yA*-disrupted mutants produce yellow spore pigments. (B)Confirmation of transformation strains by using diagnostic PCR. (C)The metabolite profiles of genetic manipulation strains using LC/MS analysis.

We further elucidate the structures of the compound 1 and 2. Compound 1 that with the molecular weight of (m/z 314 M–H) and compound 2 that with the molecular weight of (m/z 466 M–H) were detected separately in our liquid chromatography/mass spectroscopy (LC/MS) analysis (Figure 6).

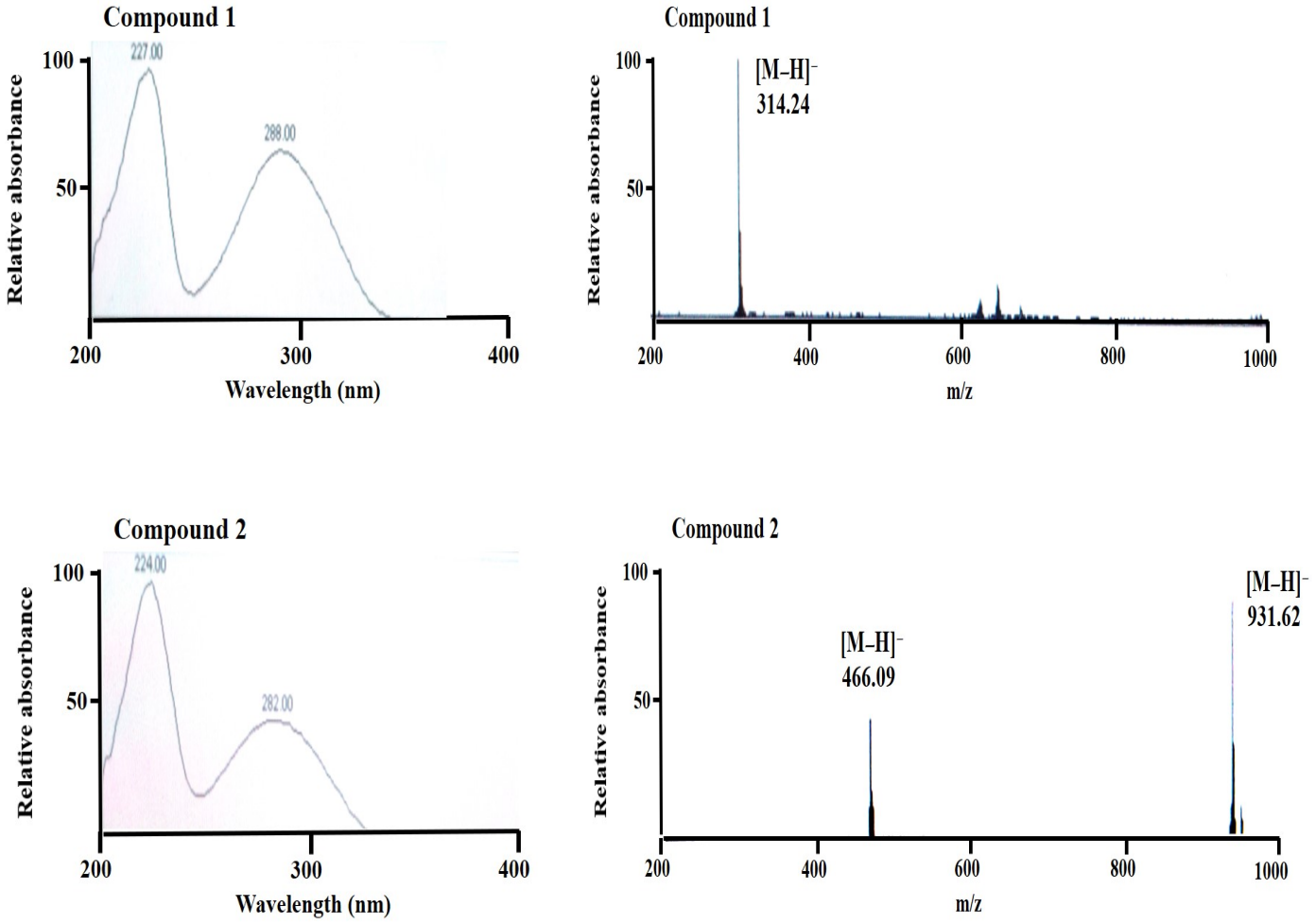

Figure 6. UV-vis and ESIMS spectra (negative mode) of the compound 1 and 2 in Mr500739.

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107年度專題研究計畫成果彙整表

計畫主持人：葉旭華	**計畫編號：**107-2635-E-041-001-
計畫名稱：利用異源系統表現紅麴菌之二次代謝產物	

成果項目					量化	單位	質化 （說明：各成果項目請附佐證資料或細項說明，如期刊名稱、年份、卷期、起訖頁數、證號...等）
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		博士級研究人員	0		
		專任人員	0		
其他成果 （無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。）					

科技部補助專題研究計畫成果自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現（簡要敘述成果是否具有政策應用參考價值及具影響公共利益之重大發現）或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估
   ■達成目標
   □未達成目標（請說明，以100字為限）
   　□實驗失敗
   　□因故實驗中斷
   　□其他原因
   說明：

2. 研究成果在學術期刊發表或申請專利等情形（請於其他欄註明專利及技轉之證號、合約、申請及洽談等詳細資訊）
   論文：□已發表　□未發表之文稿　■撰寫中　□無
   專利：□已獲得　□申請中　■無
   技轉：□已技轉　□洽談中　■無
   其他：（以200字為限）

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性，以500字為限）
   本計畫的學術成就與意義是結合生物資訊、分子生物技術與真菌培養技術並加以創新，增廣融合聚合酶連鎖反應 (fusion polymerase chain reaction)在生物技術上的應用性，另一方面有效利用小巢狀麴菌(Aspergillus nidulans)異源表現(heterologous expression)紅麴菌(Monascus ruber)之非核糖體胜肽合成酶(nonribosomal peptide synthetase)，此類酵素屬於多功能蛋白質複合體，其二次代謝產物廣泛被應用在醫療製藥界。本計畫的成果價值在於能操控紅麴菌非核糖體胜肽合成酶的表現，探討與鑑定其如何調控二次代謝產物生合成路徑，可提供具體之系統性科學佐證，以利增加紅麴菌之經濟效益與應用性。

4. 主要發現
   本研究具有政策應用參考價值：■否　□是，建議提供機關
   （勾選「是」者，請列舉建議可提供施政參考之業務主管機關）
   本研究具影響公共利益之重大發現：□否　□是
   說明：（以150字為限）