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Overexpression of an LaeA-like methyltransferase upregulates secondary metabolite production in *Aspergillus nidulans*

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Abstract

Fungal secondary metabolites (SMs) include medically valuable compounds as well as compounds that are toxic, carcinogenic and/or contributors to fungal pathogenesis. It is consequently important to understand the regulation of fungal secondary metabolism. McrA is a recently discovered transcription factor that negatively regulates fungal secondary metabolism. Deletion of *mcrA* (*mcrA*[−]), the gene encoding McrA, results in upregulation of many SMs and alters the expression of more than 1000 genes. One gene strongly upregulated by the deletion of *mcrA* is *IlmG*, a putative methyl transferase related to LaeA, a major regulator of secondary metabolism. We have artificially upregulated *IlmG* by replacing its promoter with strong constitutive promoters in strains carrying either wild-type *mcrA* or *mcrA*[−]. Upregulation of *IlmG*, on various media, resulted in increased production of the important toxin sterigmatocystin and compounds from at least six major SM pathways. *IlmG* is, thus, a master SM regulator. *mcrA*[−] generally resulted in greater upregulation of SMs than upregulation of *IlmG*, indicating that the full effects of *mcrA* on secondary metabolism involve genes in addition to *IlmG*. However, the combination of *mcrA*[−] and upregulation of *IlmG* generally resulted in greater compound production than *mcrA*[−] alone (in one case more than 460 times greater than the control). This result indicates that deletion of *mcrA* and/or upregulation of *IlmG* can likely be combined with other strategies for eliciting SM production to greater levels than can be obtained with any single strategy.

Graphical Abstract

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI:

Detailed structural information and NMR spectroscopic data, HPLC paired profile scans of liquid media conditions, secondary metabolite fold change tables for each culture condition, UV-Vis and ESIMS spectra of new and unknown compounds.

The authors declare no competing financial interest.

Fungal secondary metabolites (SMs) are compounds that are not essential for viability, but they provide a selective advantage to the producing fungi, often by inhibiting biologically important activities in their competitors. These inhibitory activities are, in some cases, medically useful, making fungal SMs a rich source of medically valuable compounds, and they provide structural inspiration that has allowed the production of synthetic or semi-synthetic compounds of great medical value^{1–5}. On the other hand, many fungal SMs are extremely toxic and/or important to fungal pathogenesis against humans, animals and plants^{5–11}. The sequencing of fungal genomes, coupled with extensive studies of SM production in cultured fungi, has revealed that fungal genomes contain many clusters of genes that encode enzymes of fungal SM biosynthetic pathways. The expression of the genes of these clusters is usually coordinately regulated. The vast majority of fungal SM biosynthetic gene clusters (BGCs) are not expressed under normal laboratory growth conditions and in order to exploit the greater fungal secondary metabolome (all of the SMs produced by all 1,000,000+ species of fungi), procedures need to be developed to activate these silent, cryptic BGCs. While a great deal of progress has been made in this area^{12–18}, much remains to be learned. In addition, understanding the regulation of fungal secondary metabolite production is important in understanding and combatting fungal pathogenesis.

McrA is a transcription factor that is a conserved master negative regulator of secondary metabolite production¹⁹. Deletion of *mcrA* in *Aspergillus nidulans*, the organism in which it was discovered, or its homologs in other fungi results in upregulation of many SMs¹⁹. At the level of transcription, deletion of *mcrA* significantly alters the expression of 1352 genes, upregulating 623 genes (including many genes of SMBGS) at least 5X and downregulating 99 genes more than 5X. The fact that *mcrA* affects the levels of transcription of so many genes raises the question of how it exerts its effects on secondary metabolism. It may affect expression of some SM genes directly and affect the expression of others by regulating their regulators.

With respect to the second possibility, we were intrigued by the fact that deletion of *mcrA* increased expression of *llmG* (AN5874 using the AspGD/FungiDB gene designation (<http://www.aspgd.org/> and <https://fungidb.org/fungidb/> respectively) 12.21 X (supplemental spreadsheet 2 in reference 19). *llmG* has been identified as one of several genes encoding LaeA-like putative methyl transferases²⁰. LaeA is a master positive regulator of secondary metabolism²¹ as part of a complex with VeA and VelB²⁰. *llmG* was transcribed at low levels and deletion of *llmG* had little impact on production of the important SM sterigmatocystin²⁰. These results left open the possibility, however, that LlmG is a positive regulator of secondary metabolism and the conditions used in the previous study were not conducive to *llmG* transcription. We have consequently artificially upregulated LlmG by replacing its promoter with the strong constitutive *gpdA* promoter²², as well as a strong constitutive

hybrid promoter based on the *nmtA* gene (AN8009). We find that upregulation of *LlmG* results in increased production of secondary metabolites from the sterigmatocystin, terrequinone A, nidulanic acid, cichorine, and emodin/monodictyphenone/prenyl xanthone pathways under the conditions we employed. This result indicates that *LlmG* is a master positive regulator of SM BGCs in *A. nidulans*. The levels of production, however, were higher in a wild-type *llmG* strain carrying an *mcrA* deletion. Since deletion of *mcrA* results in upregulation of *llmG*, the effects of *McrA* on secondary metabolite production are likely mediated, in part, through regulation of *llmG*, but the full effects of *mcrA* on secondary metabolite production involve additional genes. Finally, combining overexpression of *llmG* and deletion of *mcrA* resulted in even higher levels of SM production. This suggests that the strategy of combining the *mcrA* deletion with other strategies for eliciting SM production may be more effective than the individual strategies alone.

RESULTS AND DISCUSSION

***LlmG* is a positive regulator of secondary metabolite production.**

To study the effects of *llmG* overexpression on SM production, we used a gene targeting technique (Figure 1) to replace the promoter of *llmG* (at its chromosomal locus) with two different strong constitutive promoters, *gpdA*(p)²² and a hybrid promoter, that we designate *hnmtA*(p), created by deleting the riboswitch of the *nmtA* promoter (AN8009) and adding a short sequence from the *gpdA* promoter immediately upstream of the start codon (C. Jenkinson, T. Akashi and B. Oakley, in preparation). We anticipated that the data with the two promoters would confirm each other and, thus, rule out the possibility that results could be due to unknown characteristics of the promoters. We engineered two sets of *llmG* overexpression strains (Table 1) with the first set using LO1362 (=TN02A7²³) as the parental strain, and the second set using an *mcrA* (AN8694) deletion strain, LO8158¹⁹, as the parental strain. For purposes of media consistency, LO1362 and LO8158 were transformed with the *AtpyrG* gene replacing the mutant *pyrG89* gene and generating the pyrimidine prototrophic control strains LO11174 and LO11177, respectively.

We examined the effects of *llmG* overexpression, deletion of *mcrA*, and the combination of *llmG* overexpression and *mcrA* deletion on SM production in solid GMM [GMM(s)], liquid GMM [GMM(l)], YAG and YG. SMs were extracted from agar plates or liquid culture media as described in the experimental section and analyzed by HPLC-diode array detector (DAD)-MS. Many SMs from *A. nidulans* have previously been characterized, allowing us to identify many of the compounds produced in this study by comparing their HPLC retention time, UV-Vis absorbance, and mass spectra to those of previously identified compounds.

We first analyzed the metabolites produced by the *gpdA*(p)*llmG* strain and the *hnmtA*(p)*llmG* strain compared to the control strain, LO11174, cultivated on GMM plates (Figure 2A, i and ii). Similar results were obtained with both the *gpdA*(p)*llmG* strain (LO10860) and the *hnmtA*(p)*llmG* strain (LO10864). HPLC traces from the *gpdA*(p)*llmG* strains are shown in Figure 2. Fold change values of relative SM peak areas for strains carrying each promoter are in Supplemental Table 1. Ten metabolites were identified in the LO11174 control strain, including: sterigmatocystin (**1**), metabolites from the austinol pathway²⁴; neo-austinone (**2**), austinol (**3**), dehydroaustinol (**4**), and austinolide (**5**),

terrequinone A (**6**)²⁵, emericellin (**7**)²⁷, and three unknowns (**8-10**) (Figure 2A i). Structures are shown in Figure 3. In addition to compounds **1-10**, ten additional metabolites (**11-20**) were detected in the *IlmG* overexpression strains (Figure 2A ii and Supplemental Table 1). Metabolites from the sterigmatocystin pathway; 3'-hydroxyversiconol (**11**), versiconol (**12**), nidurufin (**13**)²⁸, averufin (**14**)²⁸, and three putative sterigmatocystin intermediates or shunt products, unknowns (**15-17**)¹⁹, were upregulated in the *IlmG* overexpression strains compared to LO11174, along with nidulanin A and its derivatives (**18-20**)^{13,19}. While sterigmatocystin (**1**), terrequinone (**6**) and emericellin (**7**) were detected in LO11174, these compounds were upregulated in the *IlmG* overexpression strains along with compounds (**11-20**). The same compounds that were upregulated in the *IlmG* overexpression strains were also upregulated in the *mcrA* strain (LO11177), the *mcrA*, *gpdA(p)IlmG* strain (LO10881) (Figure 2A, iii and iv and Supplemental Table 1) and the *mcrA*, *hnmA(p)IlmG* strain (LO10868) (Supplemental Table 1), but to a much greater degree. Furthermore, while the deletion of significantly enhanced SM upregulation compared to the overexpression of *IlmG*, strains carrying both *mcrA* and *IlmG* overexpression constructs produced even greater amounts of SMs than the strain carrying *mcrA* alone (LO11177) (Supplemental Table 1). We generally saw greater levels of upregulated SMs in strains carrying *gpdA(p)IlmG* (LO10860 and LO10881) than in equivalent *hnmA(p)IlmG* strains (LO10864 and LO10868) although the difference was often slight and there were exceptions (Supplemental Table 1).

***IlmG* overexpression results in the upregulation of several additional SMs under different culture conditions.**

Many have reported that fungi, like other microbes, produce different SMs under different growth conditions²⁹. We were curious as to whether the overexpression of *IlmG* in different culture media would result in the production of additional SMs. We consequently analyzed the metabolites produced by the *IlmG* overexpression strains cultivated on YAG plates in comparison with LO11174 (Figure 2B, i and ii, Supplemental Table 2). Three metabolites were identified in the LO11174 control strain, including, 2,ω-dihydroxyemodin (**21**)²⁷, 3-methylorsellinic acid (**22**), and an unknown compound (**23**). Compounds **21-23** were upregulated in the *IlmG* overexpression strains along with twelve additional metabolites (**1, 14, 18-19** and **24-31**), including sterigmatocystin (**1**) and its intermediates; averufin (**14**)²⁸, versicolorin B(**24**)²⁸, averantin (**25**)³⁰, metabolites from the emodin/mondictyphenone pathway³¹; mondictyphenone (**26**), ω-hydroxyemodin (**27**), 2-hydroxyemodin (**28**), emodin (**29**), and chrysophanol (**30**), nidulanins (**18** and **19**)^{13,19}, 3-methylorsellinic acid (**22**)³², and an unknown (**31**) (Figure 2B, ii-iv). As was the case for GMM(s), we observed an even greater increase in production of the same SMs in the *mcrA* strain than in the *IlmG* overexpression strains, and, notably, the *gpdA(p)IlmG*, *mcrA* strain demonstrated enhanced SM production in comparison with the *mcrA* strain (Figure 2B, iii and iv, Supplemental Table 2).

Finally, we analyzed the metabolites produced in liquid media by the *IlmG* overexpression, *mcrA*, and double mutant strains in comparison with LO11174, GMM(I) and YG (GMM and YAG without agar) (Supplemental Figure 1A, i-iv, Supplemental Figure 1B, i-iv and Supplemental Tables 3 and 4). Similar to GMM(s), in the *IlmG* overexpression and *mcrA*

strains, we observed upregulation of SMs from the sterigmatocystin, terrequinone A, and nidulanin A pathways in GMM(l), as well as metabolites from the cichorine pathway (Supplemental Figure 1A, i-iv). Additional putative sterigmatocystin intermediates or shunt products were detected in GMM(l), including unknowns (35-38)¹⁹, along with an additional nidulanin A derivative (32)^{13,19}, and cichorine pathway metabolites; O-methyl-3-methylorsellinaldehyde dimer (39)¹⁹ and cichorine (40)³³ (Supplemental Figure 1A i-iv and Supplemental Table 3). Metabolites from the same pathways were upregulated in the *llmG* overexpression and *mcrA* strains when cultured in YG compared to YAG, including the monodictyphenone/emodin, nidulanin A and cichorine pathways, while no sterigmatocystin-related compounds were detected under this condition (Supplemental Figure 1B i-iv and Table S4). In the *gpdA(p)llmG* and *hnmA(p)llmG* strains, we detected the emodin intermediate, atrochrysone (44)¹⁵, a nidulanin A derivative (45)^{13,19}, and a cichorine intermediate, nidulol (46)³³ (Supplemental Figure 1B i and ii and Supplemental Table 4). In the *mcrA* , the *mcrA* , *gpdA(p)llmG* and *hnmA(p)llmG* strains we observed the production of emodin derivatives [(cis/trans)-emodin-physicon bianthrone (47 and 48)³⁴, and four unknowns (49-53) (Supplemental Figure S1B iii and iv and Supplemental Table 4)]. Relative to the control strain, LO11174, we observed as much as 98 times greater production of some of these unknowns (49-53) in the *gpdA(p)llmG* strain, up to 435 times greater production in the *mcrA* strain and more than 460 times greater production in the *gpdA(p)llmG*, *mcrA* strain (Supplemental Table 4). We should note that metabolites from the F-9775A/B pathway^{31,32} [orsellinic acid (41), F-9775A (42), and F-9775B (43)] were detected in the control strain (LO11174), *llmG* overexpression strains, an *mcrA* strain and *mcrA* , *llmG* overexpression strains at relatively consistent levels (Supplemental Figure S1B ii-iv and Supplemental Table 4). Also, although these metabolites are difficult to detect with the DAD, analysis of the MS data indicated the production of multiple emericellamides in varying media conditions, whereas the overexpression of *llmG* or the deletion of *mcrA* did not affect the level of production of these compounds (Supplemental Tables 1, 3 and 4).

The results of the SM production analysis with both solid and liquid media indicate that *LlmG* is a positive regulator of SM production that is responsible for upregulating metabolite production from the sterigmatocystin, terrequinone A, nidulanin A, monodictyphenone/emodin and prenyl xanthone pathways. The austinol, F-9775A/B, and emericellamide pathways are not affected by either the overexpression of *llmG* or the deletion of *mcrA* . While overexpression of *llmG* increases production of SMs from the pathways listed above, the deletion of *mcrA* results in even greater levels of SM production. This observation suggests that the increased expression of *llmG* resulting from the deletion of *mcrA* is only partially responsible for the increased SM production observed in *mcrA* strains, and that other genes must play a part in fully upregulating SM production. Importantly, for many compounds, strong constitutive expression of *llmG* in combination with deletion of *mcrA* resulted in higher production (and in some cases much higher production) than the deletion of *mcrA* or constitutive overexpression of *llmG* alone. This was particularly true in liquid media (Tables S3 and S4). Since *LlmG* is a putative methyl transferase related to *LaeA*, we speculate that *LlmG* is involved in the methylation of chromatin associated with certain BGCs. Increased expression of *llmG* would result in increased methylation of chromatin in these regions, which would allow increased

transcription. Deletion of *mcrA* increases *IlmG* transcription and might also increase the expression of transcription factors required for transcription of these BGCs, and/or increase expression of primary metabolism genes required for production of SM precursors. These factors, in combination, would account for the greatly increased SM production from these BGCs. It follows that increasing *IlmG* expression further in an *mcrA* deletion by driving its transcription with a strong constitutive promoter further increases chromatin methylation and allows even greater transcription and, consequently, SM production.

Identification of unknown (23) as an intermediate of cichorine biosynthesis.

Earlier work developing methods to delete entire *A. nidulans* SM clusters facilitated the engineering of a genetic dereplication strain LO8030^{15,26}. Most of the SM gene clusters often expressed in *A. nidulans* were deleted in LO8030, and the SMs produced by these deleted clusters include: sterigmatocystin, the emericellamides, asperfuranone, monodictyphenone, terrequinone, F9775A and B, asperthecin, austinol and dehydroaustinol²⁶. These deletions provide a greatly reduced SM background making it easier to detect SMs from other clusters. We questioned whether unknowns (23 and 31), produced in significant amounts in strains carrying an *mcrA* deletion, were potentially new compounds as their identities could not be determined based on UV-absorbance, MS, and retention time data alone. In an earlier study¹⁹ AN8694 was deleted in strain LO8030 to create strain LO8112. In this investigation, using LO8112 as a parental strain, we replaced the *IlmG* promoter with the *gpdA* promoter creating strain LO11505. Culturing LO11505 on YAG plates, we observed enhanced production of unknown (23) compared to strain LO10881 (*gpdA(p)IlmG, mcrA* with SM clusters intact), while the production of unknown (31) was completely abolished (Figure 4A, i and ii). This indicated that unknown (31) must belong to one of the pathways that were deleted when engineering strain LO8030. To characterize unknown (23), we isolated the compound from a large-scale culture of LO11505 (see Experimental Procedures). The structure of 23, elucidated from NMR spectroscopic data (Supplemental Figures 3 and 4), is shown in Figure 4B. While unknown (23) is a compound not previously reported, based on its chemical structure it is likely to be an intermediate or shunt product of the cichorine biosynthetic pathway³³. We propose a biosynthetic pathway for cichorine that includes unknown (23) in Supplemental Figure 6.

CONCLUSION

We constructed *IlmG* overexpression strains and compared their SM profiles to a control strain lacking *IlmG* overexpression in varying culture conditions. We also compared SM production levels of these strains to *mcrA* and combined *mcrA* and *IlmG* overexpression strains. We observed that *IlmG* overexpression increased the production of metabolites from the sterigmatocystin, terrequinone A, nidulanin A, cichorine, monodictyphenone/emodin and prenyl xanthone pathways, while not affecting SM production from the austinol, F-9775A/B, and emericellamide pathways. The increased production of multiple SMs from several BGCs indicates that *IlmG* is a master SM regulatory gene. One of the upregulated metabolites, unknown (23), was characterized and determined to be a compound not previously reported that is a putative intermediate or shunt product of the cichorine biosynthetic pathway. The deletion of *mcrA* had a greater impact on SM production than

IlmG overexpression alone. This result highlights how regulatory genes in addition to *IlmG* are likely to be involved in generating the production of SMs at levels that are observed when *mcrA* is deleted. The combination of overexpression of *IlmG* and deletion of *mcrA*, however, resulted in even greater metabolite production than *mcrA* alone. This result raises the exciting possibility that deletion of *mcrA*, and, perhaps, overexpression of *IlmG*, can be used in combination with other strategies to elicit greater SM production than any of these strategies can produce alone and, thereby, facilitate the discovery and production of valuable new SMs.

METHODS

Molecular Genetic Procedures.

Transformation procedures and production of linear molecules for transformations by fusion PCR were as previously described^{23,35,36}. Strains LO1362, LO8112 and LO8158, which carry *pyrG*⁸⁹, were transformed to *pyrG*⁺ to produce strains LO11172–11174, LO11510–LO11514, and LO11175–LO11177 respectively. The transforming fragment was created by fusion PCR and consisted of a 1006 bp fragment upstream of *AnpyrG* (AN6157), a 1491 bp fragment carrying the *Aspergillus terreus pyrG* gene (ATEG_09675) and a 978 bp fragment 3' to *AnpyrG*. The upstream fragment was amplified with primers LizP6853 and LizP6855, the *AtpyrG* fragment was amplified from plasmid pLO103³⁷ with primers LizP2018 and LizP2019 and the 3' fragment was amplified with primers LizP6856 and LizP6858. Fusion PCR was carried out with nested primers LizP6854 and LizP6857. *pyrG*⁺ transformants were selected on YAG medium without uridine and uracil. Transformants were verified by three diagnostic PCR's using the following primer combinations: LizP6852 and LizP6859 (both are outside the transforming fragment), LizP6852 and 4139 (LizP4139 is a reverse primer inside the *AtpyrG* gene), and LizP6398 and 6858 (LizP6398 is a forward primer inside the *AtpyrG* gene). Primers are listed in Supplementary Table 5.

The *gpdA* promoter was amplified from genomic DNA using forward primer LizP4800 (which has a 'tail' to facilitate fusion PCR) and reverse primer LizP2273, to produce a 1231 bp fragment that is immediately 5' to the *gpdA* start codon. The fragment that was used to replace the promoter of *IlmG* (AN5874) with the *gpdA* promoter was created by fusion PCR. It consisted of 1) a 1080 bp fragment upstream of *IlmG* amplified with primers LizP7021 and LizP7023, 2) the *A. terreus pyrG* fragment described above, 3) the *gpdA* promoter fragment and 4) a 1141 bp fragment starting with the start codon from *IlmG* (amplified with primers LizP7025 and LizP7027). Nested primers LizP7022 and LizP7026 were used for the fusion PCR reaction, creating a 4.7 kb DNA fragment. This fragment was used to transform host LO1362 (creating LO10860), host LO8158 (creating LO10881) and host LO8112 (creating LO11505). Transformants were verified using the following primer pairs: LizP7020 and LizP7028, which are upstream and downstream of *IlmG* and outside of the transforming fragment, LizP7020 and LizP4139 or LizP6399 (LizP4139 and LizP6399 are reverse primers within the *AtpyrG* gene) and LizP6398 (forward primer within *AtpyrG*) and LizP7028. The replacement of the *IlmG* promoter with a hybrid *nmtA* promoter was carried out using the same strategy.

Culturing and HPLC-DAD-MS Analysis.

For agar plate cultures, *A. nidulans* strains were incubated at 37°C on GMM(s) (10 g L⁻¹ D-glucose, 6 g L⁻¹ NaNO₃, 0.52 g L⁻¹ KCl, 0.52 g L⁻¹ MgSO₄·7H₂O, 1.52 g L⁻¹ KH₂PO₄, 15 g L⁻¹ agar, and 1 ml L⁻¹ Hutner's trace element solution³⁸), or YAG (5 g L⁻¹ yeast extract, 20 g L⁻¹ D-glucose, 15 g L⁻¹ agar, and 1 ml L⁻¹ Hutner's trace element solution³⁸) plates supplemented with riboflavin (2.5 mg L⁻¹) and/or pyridoxine (0.5 mg L⁻¹) when necessary. Plates were inoculated with 1.0×10^7 spores per 10-cm plate. After 3 days, three plugs (7-mm diameter) were cut out and transferred to a 7 ml screw-cap vial. The material was extracted with 3 ml of methanol followed by 3 ml of 1:1 dichloromethane-methanol with 1 hr of sonication. The extract was transferred to a clean vial and the solvent was evaporated by TurboVap LV (Caliper LifeSciences). The residues were re-dissolved in 5 ml of EtOAc and 5 ml of water, the EtOAc layer was collected and the solvent was evaporated by TurboVap LV. The crude extract was re-dissolved in 0.3 ml of DMSO:MeOH (1:4) and 10 µl was injected to LC-DAD-MS analysis as previously described³¹.

For liquid culture, 3×10^7 spores were grown in 30 ml GMM(l) or YG liquid medium (recipes same as above except no agar was added) in 125 ml Erlenmeyer flasks at 37°C with shaking at 180 rpm. After 5 days, culture media and hyphae were collected by filtration. Culture media were extracted with an equal volume of EtOAc. In order to extract the most acidic phenolic compounds, the water layer was extracted with an equal volume of EtOAc again after acidification (pH = 2). Both EtOAc extracts were then evaporated by TurboVap LV. The residues were re-dissolved in 0.5 ml of DMSO:MeOH (1:4) and analyzed by LC-DAD-MS as described above.

LC/MS spectra were obtained using a ThermoFinnigan LCQ Advantage ion trap mass spectrometer with a reverse phase C₁₈ column (Alltech Prevail C₁₈; particle size, 3 µm; column, 2.1 by 100 mm) at a flow rate of 125 µL min⁻¹. The solvent gradient for LC/MS was 5% MeCN–H₂O (solvent A) and 95% MeCN–H₂O (solvent B), both of which contained 0.05% formic acid, as follows: 100% solvent A from 0 to 5 min, 0 to 25% solvent B from 5 min to 6 min, 25% to 100% solvent B from 6 to 35 min, 100% solvent B from 35 to 40 min, 100% to 0% solvent B from 40 to 45 min, and re-equilibration with 100% solvent A from 45 to 50 min. Conditions for MS included a capillary voltage of 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.

Compound Isolation and Purification.

LO11505 was cultivated in 25 Petri dishes (150 mm diameter) containing a total of 2 L of YAG medium supplemented with riboflavin (2.5 mg L⁻¹) for 3 days at 37°C. The agar was then chopped up and sonicated in the same manner as above. The organic material was evaporated and extracted twice with ethyl acetate. The crude material was subjected to silica gel column chromatography, using ethyl acetate and hexanes as the eluent. The material was further separated by preparative HPLC [Phenomenex Luna 5 µm C18 (2), 250 × 21.2] with a flow rate of 5.0 mL min⁻¹ and measured by a UV detector at 280 nm.

Structural Characterization.

NMR spectral data were collected on a Varian Mercury Plus 400 spectrometer. High-resolution electrospray ionization mass spectrum (HRESIMS) was obtained on Thermo Scientific Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometer with an Eclipse XDB-C₁₈ column (Agilent 5 μ m 4.6 $\times$ 150 mm) at a flow rate of 125 μ L min⁻¹. Conditions for MS included a spray voltage of 3.5 kV, sheath gas flow rate 20 au, auxiliary gas flow rate at 5 au, sweep gas flow rate at 1 au, capillary temperature at 275 °C, s-lens RF level 55, auxiliary gas heat temperature at 325 °C, scan range of 100–600 m/z, resolution 140,000, AGC target 3×10^6 , and maximum injection time of 200 ms.

7-methoxy-6-methyl-1,3-dihydroisobenzofuran-1,5-diol (23): white powder; UV $\lambda_{\text{max}}^{\text{MeOH}}$ nm: 233, 281; ¹H NMR (CD₃OD): δ = 2.06 (3H, s), 3.86 (3H, s), 4.94 (2H, dddq), 6.27 (1H, d), 6.43 (1H, s); ¹³C NMR (CD₃OD): δ = 8.9, 60.3, 72.9, 102.6, 108.0, 116.5, 119.5, 141.3, 155.9, 159.4. For UV and ESIMS spectrum, see Supplemental Figure 2; HRESIMS obtained m/z [M – H] = 195.0654 (calcd 195.0657 for C₁₀H₁₁O₄) (See Supporting Information for a detailed structural characterization of compound **23**).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Replacement of the native *IlmG* promoter with a strong constitutive promoter. A. Four fragments are separately amplified by PCR (primers are given in Supplemental Table 1). The four fragments consisted of a 1080 bp fragment amplified from upstream of the *IlmG* (AN5874) coding sequence (nt minus 1510- to nt minus 430), a 1491 bp fragment containing the *Aspergillus terreus pyrG* gene (*AtpyrG*), a fragment containing the constitutive promoter (a 1231 bp fragment in the case of the *gpdA* promoter and a 502 bp fragment in the case of the hybrid *nmtA* promoter) and a 1141 bp fragment extending from the start codon of *IlmG* into the *IlmG* coding sequence. B. The four fragments were fused together by fusion PCR using nested primers creating a transforming fragment. B and C. The fusion PCR fragment is used to transform the *A. nidulans* host strain with *AtpyrG* as the selectable marker. Double homologous recombination results in *IlmG* transcription being driven by the constitutive promoter.

Figure 2.

HPLC paired profile scans of *IlmG* overexpression and *mcrA* deletion strains compared to the control strain on A. GMM(s) and B. YAG plates. (i) Control strain (LO11174), (ii) *gpdA(p)IlmG* strain (LO10860), (iii) *mcrA* strain (LO11177), and (iv) *mcrA ::gpdA(p)IlmG* strain (LO10881).

Figure 3.

Chemical structures of compounds that were upregulated in the *gpdA(p)llmG* (LO10860, LO10864), *mcrA* (LO11177) and *gpdA(p)llmG::mcrA* (LO10868, LO10881) strains. Sterigmatocystin (1), terrequinone A (6), emerellin (7), 3'-hydroxyversiconol (11), versiconol (12), nidurufin (13), averufin (14), nidulanin A (18), 2,ω-dihydroxyemodin (21), 3-methylorsellinic acid (22), cichorine intermediate (23), versicolorin B (24), averantin (25), monodictyphenone (26), ω-hydroxyemodin (27), 2-hydroxyemodin (28), emodin (29), chrysophanol (30), O-methyl-3-methylorsellinaldehyde dimer (39), cichorine (40), atrochrysone (44), nidulol (46), trans-emodin-physicon bianthrone (47), cis-emodin-physicon-bianthrone (48).

Figure 4.

Enhanced production of compound **23** observed in a multicluster deletion, *mcrA* , *gpdA(p)llmG* strain. A. (i) HPLC profile of a *mcrA* , *gpdA(p)llmG* strain (LO10881) on YAG, indicating the production of unknown **23**. (ii) HPLC profile, on YAG, of LO11505 in which *mcrA* was deleted and *llmG* was upregulated in strain LO8030, that was engineered to have multiple SM gene clusters deleted. Enhanced production of compound **23** was observed in LO11505 compared to LO10881. B. The structure of compound **23**. Based on structural similarities observed between compound **23** and cichorine (**41**), we predict that compound **23** is an intermediate of the cichorine biosynthetic pathway.

Table 1.

Strains used in this study

Strain	Genotype	Reference
FGSCA442	<i>facB101, riboB2, chaA1, sE15, nirA14</i>	Fungal Genetics Stock Center
LO1362	<i>pyroA4, riboB2, pyrG89, nkuA::argB</i>	[²³]
LO8158	<i>pyroA4, riboB2, pyrG89, nkuA::argB, mcrA::AfpyroA</i>	[¹⁹]
LO8030	<i>pyroA4, riboB2, pyrG89, nkuA::argB, ste(AN7804-AN7825), eas(AN2545-AN2549), afc(AN1036-AN1029), mdp(AN10023-AN10021), tdt(AN8513-AN8520), aus(AN8379-AN8384, AN9246-AN9259), ors(AN7906-AN7915), apt(AN6000-AN6002)</i>	[²⁶]
LO8112	<i>mcrA::AfpyroA</i> in LO8030 (note: a sister transformant of LO8111)	[¹⁹]
LO11172-LO11174	<i>pyrG89::AtpyrG</i> in LO1362	This work
LO11175-LO11177	<i>pyrG89::AtpyrG</i> in LO8158	This work
LO10859-LO10860	<i>AtpyrG-gpdA(p)llmG</i> in LO1362	This work
LO10863-LO10864	<i>AtpyrG-hmmA(p)llmG</i> in LO1362	This work
LO10867-LO10868	<i>AtpyrG-hmmA(p)llmG</i> in LO8158	This work
LO10881-LO10882	<i>AtpyrG-gpdA(p)llmG</i> in LO8158	This work
LO11505-LO11509	<i>AtpyrG-gpdA(p)llmG</i> in LO8112	This work
LO11510-LO11514	<i>pyrG89::AtpyrG</i> in LO8112	This work