An Efficient System for Heterologous Expression of Secondary Metabolite Genes in *Aspergillus nidulans*

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Abstract

Fungal secondary metabolites (SMs) are an important source of medically valuable compounds. Genome projects have revealed that fungi have many SM biosynthetic gene clusters that are not normally expressed. To access these potentially valuable, cryptic clusters, we have developed a heterologous expression system in *Aspergillus nidulans*. We have developed an efficient system for amplifying genes from a target fungus, placing them under control of a regulatable promoter, transferring them into *A. nidulans* and expressing them. We have validated this system by expressing non-reducing polyketide synthases of *Aspergillus terreus* and additional genes required for compound production and release. We have obtained compound production and release from six of these NR-PKSs and have identified the products. To demonstrate that the procedure allows transfer and expression of entire secondary metabolite biosynthetic pathways, we have expressed all the genes of a silent *A. terreus* cluster and demonstrate that it produces asperfuranone. Further, by expressing the genes of this pathway in various combinations, we have clarified the asperfuranone biosynthetic pathway. We have also developed procedures for deleting entire *A. nidulans* SM clusters. This allows us to remove clusters that might interfere with analyses of heterologously expressed genes and to eliminate unwanted toxins.

Introduction

Fungal secondary metabolites (SMs) have biological activities that make them a rich source of medically useful compounds.[1–4] Sequencing of fungal genomes has revealed that many fungi contain large numbers of genes involved in secondary metabolism and that the genes of individual SM biosynthetic pathways are clustered together.[3, 5–7] The number of SM biosynthetic clusters is generally much larger than the number of SMs known to be produced by the organism[3] because the majority of fungal SM clusters are silent under most conditions. There is relatively little overlap in SM clusters even among closely related fungi,[3, 5] indicating that fungi, collectively, have the ability to produce an extraordinary number of novel compounds of potential medical value. Scores, if not hundreds, of fungal genomes will be sequenced in coming years and the SM gene clusters within them will constitute a remarkable medical resource if they can be accessed efficiently.

Perhaps less obviously, identifying and obtaining production of early intermediates in fungal SM pathways is also extremely valuable. They promise to be excellent starting points for combinatorial chemistry to produce compounds of potential medical value[8] because they occupy a wider chemical space than synthetic combinatorial libraries and they are more drug-like.[9–11] Early intermediates in polyketide biosynthetic pathways in particular offer, in principle, a platform for synthetic chemistry (medicinal and non-medicinal) that is sustainable and efficient. For example, Somoza et al. recently reported the synthesis of lipoxygenase inhibitors in a very small number of steps from an azaphilone intermediate obtained by reengineering a biosynthetic pathway of *A. nidulans*.[12] To realize the potential of fungal secondary metabolites, it is important to be able to identify the products of fungal biosynthetic pathways and intermediates in those pathways, particularly the early intermediates released from polyketide synthases (PKSs) and non-ribosomal peptide synthetases (NRPSs).

For the few species with good molecular genetic systems, progress is being made in using molecular genetic tools to up-regulate cryptic clusters, determine the products of the clusters and decipher the biosynthetic pathways encoded by the clusters.[13, 14] Most fungi, however, have poor or nonexistent molecular genetic systems, and this approach is not feasible.

A substantial number of SM gene clusters are regulated, at least in part, at the chromatin level[15] and one useful approach for obtaining SM production from fungi without molecular genetic systems is to treat them with compounds that inhibit histone deacetylases or methyl transferases. These alter acetylation or methylation of histones resulting in alteration of chromatin packing and can lead to expression of SMs from cryptic clusters.[16–20] This approach is often called chemical epigenetics. While the approach is useful in eliciting production of compounds it is limited in at least two important ways. First, only a portion of SM pathways are activated by this approach[20] and second, this approach, unlike genetic approaches, doesn't facilitate the production of useful intermediates or the deciphering of the biosynthetic pathways for the compounds produced.

Another solution is to develop heterologous expression systems. There has been some success in heterologous expression of fungal genes in other fungi,[13, 14, 21–29] but in each case a great deal of work was required to obtain expression of one or a few genes.

We have taken two major steps toward the development of the model ascomycete *Aspergillus nidulans* as a system for heterologous expression of fungal SMs. First, we have developed efficient procedures for deleting entire SM gene clusters to prevent production of toxic or otherwise unwanted compounds. Second, we have developed a rapid, robust and efficient approach, using fusion PCR to amplify genes from a target fungus, place them under the control of the regulatable *alcA* promoter [*alcA*(p)][30, 31] (Table 1) and transfer

them into *A. nidulans*. The approach includes marker recycling so that an unlimited number of genes can potentially be transferred into a single strain without running out of selectable markers. We have used this approach to transfer all of the non-reducing polyketide synthase (NR-PKS) genes of *Aspergillus terreus* into *A. nidulans* along with additional genes required for production or release of the NR-PKS products. This has allowed us to isolate and identify the products of six *A. terreus* NR-PKS genes. To determine if it is practical to use this approach to express an entire SM pathway, we have transferred all the genes of a putative *A. terreus* azaphilone biosynthetic pathway into *A. nidulans*. We find that it produces asperfuranone, and we have obtained useful yields of this compound. The ability to express genes of this pathway in various combinations in *A. nidulans* has allowed us to analyze the asperfuranone biosynthetic pathway and improve our understanding of asperfuranone biosynthesis.

Results and Discussion

Deletion of entire *A. nidulans* SM gene clusters

An important step in developing *A. nidulans* as a heterologous expression system is to eliminate production of the most abundant *A. nidulans* SMs to reduce the SM background and facilitate detection and purification of the products of heterologously expressed clusters. This may also reduce competition for substrates such as malonyl-CoA, and enhance the yield of heterologously expressed SMs. While deleting a key gene in a pathway can eliminate production of the final product of the pathway, other pathway genes will still be expressed and may modify intermediates or final products of heterologously expressed gene clusters. We consequently developed approaches to delete entire SM clusters.

Our first strategy was to try to replace an entire cluster with a single selectable marker. We targeted the 25-gene cluster that produces sterigmatocystin (ST), an abundant toxin,[32] for deletion by replacing it with the *Aspergillus fumigatus pyrG* (*AfpyrG*) gene[33](Table 1 and Figure 1a). For this strategy to be successful, the flanking sequences on the transforming molecule must undergo homologous recombination with two chromosomal sequences > 50 kb apart. The target strain carried the mutation *pyrG*89 which blocks growth on media unsupplemented for pyrimidines. *AfpyrG* complements *pyrG*89, allowing growth in the absence of added pyrimidines. To reduce non-homologous recombination, we used a strain carrying *nkuA* Δ.[34] To evaluate the efficiency of cluster replacement, the strain also carried *riboB2* (riboflavin requirement) and a replacement of the *stcJ* gene, a gene within the ST cluster, with the *A. fumigatus riboB* gene (*AfriboB*).[34] *AfriboB* complements *riboB2* and supports growth in the absence of riboflavin. Correct homologous recombination of the transforming fragment with the genome would result in excision and loss of the entire ST cluster including the *AfriboB* gene inserted in place of *stcJ*. Loss of *AfriboB*, in turn, results in a riboflavin requirement. Riboflavin auxotrophy is, thus, an indication that at least the portion of the ST cluster with the inserted *AfriboB* gene has been deleted. Transformation yielded abundant *AfpyrG*+ transformants. We tested 118 transformants and found that 78 required riboflavin. At least a portion of the ST cluster was, thus, replaced in 66% of transformants. We tested seven of the riboflavin-requiring transformants by diagnostic PCR (Supplemental Figure S1) and found that the entire ST cluster had been replaced by *AfpyrG* in each case. The deletion of the ST cluster was further confirmed by Southern hybrization for one transformant (result not shown). The size of the fragment deleted was 50,096 bp and these data, thus, reveal that even a very large SM cluster can be easily deleted by replacement with a small DNA fragment carrying a selectable gene. They also indicated that the procedure was efficient enough that it was not necessary to embed a selectable marker such as *AfriboB* within the cluster to identify cluster deletants.

We next deleted the emericellamide cluster[35] using the same approach except that we used 1 kb flanking sequences and we did not embed a marker within the cluster. The size of the region targeted for deletion was 37,423 bp. Five of eight transformants tested by diagnostic PCR had correct deletions of the cluster. Deletion of the cluster was further confirmed for one of these transformants by Southern hybridization. For clusters of this size, 1 kb flanking sequences are, thus, perfectly adequate.

Because we have previously inserted *AfpyrG* into many SM clusters, we developed a second approach that allows us to delete these clusters while recycling the *AfpyrG* marker so that it can be used again (Figure 1b). *AfpyrG* encodes orotidine-5'-phosphate decarboxylase, a key enzyme in pyrimidine metabolism. If 5-fluoro-orotic acid (5-FOA) is present, orotidine-5'-phosphate decarboxylase converts it to the toxic compound 5-fluorouracil. 5-FOA can thus be used to select for the loss of genes such as *AfpyrG* that encode a functional orotidine-5'-phosphate decarboxylase.[36, 37] We attempted to delete a very large (77 kb) cluster containing the NRPS gene AN9226 (product unknown) into which we had previously inserted *AfpyrG*. The transforming fragment contained approximately 2 kb of flanking DNA from each side of the cluster but no selectable marker and the actual size of the region targeted for deletion was 77,070 bp. Diagnostic PCR revealed that five of nine transformants selected on 5-FOA carried the correct cluster deletion. We also deleted the emericellamide cluster again using this approach with similar results. Since, in this procedure, *AfpyrG* is removed, it can be inserted into another cluster and the process repeated to delete, in principle, an unlimited number of clusters.

Finally, we developed a variant of the loop-out recombination procedure developed for *Aspergillus oryzae* and *Aspergillus sojae*[38] (Supplementary Figure S2). We used fusion PCR to create the transforming fragment rapidly, rather than the more complex and time-consuming published procedure,[38] and we used *AfpyrG* as the selectable marker. With this procedure we deleted the ST, emericellamide, orsellinic acid/F9775A, B,[39,40] asperfuranone, monodictyphenone[41] and terrequinone[42] clusters. In summary, we have developed three approaches that allow us to delete entire SM gene clusters in *A. nidulans* using linear DNA molecules created rapidly by fusion PCR. The ability to delete entire *A. nidulans* SM clusters allows us to eliminate production of undesired toxins and to eliminate clusters that might interfere with the analysis of heterologously expressed SM pathway genes. This removes important barriers in developing *A. nidulans* as a production organism.

An efficient procedure for heterologous expression of SM genes in *A. nidulans*

The approach we have developed uses fusion PCR to amplify genes from genomic DNA of a target fungus, place them under control of *alcA*(p) and fuse them into fragments that can be used to transform *A. nidulans* (Figure 2). Because core SM genes such as PKS or NRPS genes are large and difficult to amplify by PCR, we have developed a system in which we create two (or, for very large genes, three) smaller transforming fragments that fuse by homologous recombination *in vivo* during transformation resulting in reconstruction of a full length coding sequence under control of *alcA*(p) (Figure 2). We use flanking sequences from the *wA* locus to direct integration such that the *wA* gene is replaced with the transforming fragment and this results in correct transformants having white conidia. Each of the transforming fragments carries a selectable marker (*AfpyrG* and *AfriboB* in the example shown) and we select for transformants that are transformed with both fragments (i.e. they grow on media unsupplemented with pyrimidines or riboflavin). Correct transformants are verified by diagnostic PCR.

We transfer additional genes as shown in Figure 3. In the example shown, a second gene (Gene B) is inserted adjacent to a PKS gene that has been inserted at the *wA* locus. The procedure uses fusion PCR to create a fragment that carries 1) a targeting sequence of about

1000 bp that corresponds to the 3' end of coding sequence and 3' untranslated region of the gene next to which the fragment is to be inserted (in this case the PKS), 2) *alcA*(p), the coding region and 3' untranslated region of the gene to be transferred, 3) a selectable marker (*AfpyroA* in the example shown, and 4) approximately 1000 bp of *wA* downstream sequence. The selectable marker and the *wA* downstream sequence can be fused into a cassette that can be used for additional experiments. Integration by homologous recombination inserts Gene B and removes *AfriboB*. Transformants are selected on their ability to grow in media unsupplemented with pyridoxine, tested to determine if they require riboflavin, and verified by diagnostic PCR. The process can be repeated for a third gene using *AfriboB* and displacing *AfpyroA*. Since a selectable marker is recycled at each step, that there is always a selectable marker available for the next transformation, and, in principle, an unlimited number of genes can be transferred. An advantage of this approach is that genes do not have to be cloned in the normal sense because they are simply amplified from genomic DNA. The procedure is also rapid in that transforming fragments can be constructed in two days once PCR primers have been obtained and many transforming fragments can be constructed in parallel with one thermocycler.

One potential concern with this procedure is that since the genes that have already been transferred and the gene on the transforming fragment are both under control of *alcA*(p), homologous recombination between *alcA*(p) upstream of one or more of the previously transferred genes and *alcA*(p) on the transforming fragment could result in the deletion of one or more of the previously transferred genes. Such events are easily detectable by diagnostic PCR and in our hands they do not occur frequently enough to slow progress. We routinely test several transformants from each transformation and find that most of them are correct. The likely reason that recombination between *alcA*(p) sequences is not a significant problem is that the flanking sequences are much larger than the *alcA*(p) sequence (about 1000 bp vs 400 bp), and it is possible that the end sequences are more recombinogenic than the *alcA*(p) sequence which is internal on the transforming fragment.

Another potential concern was that PCR amplification of large genes might result in PCR-induced mutations that would render the genes dysfunctional. We consequently used high fidelity DNA polymerases and screened several transformants in each case. These steps appeared to obviate the problem although, in a few cases, we found that one transformant among several tested did not produce a compound that its sister transformants produced.

We analyzed the PKS genes in the *A. terreus* genome using the Broad Institute *Aspergillus* Comparative Database (http://www.broadinstitute.org/annotation/genome/aspergillus_group/MultiHome.html) and identified genes encoding nine NR-PKSs, 16 highly-reducing PKSs (HR-PKSs), one hybrid PKS-NRPS and one partially reducing PKS. [Designations are based on domain architectures and phylogeny (Supplementary Figure S3)]. To determine the efficacy of our approach, we focused on NR-PKS genes. We previously completed the elucidation of the chemical products released from all *A. nidulans* NR-PKSs by replacing their promoters with *alcA*(p), while coexpressing accessory genes as necessary.[43] This led us to divide NR-PKSs into seven groups, based on phylogenetic analyses, domain structures, and products released.[43] Group I ~ V NR- PKSs do not have a C-methyltransferase (CMeT) domain. Group I NR-PKSs contain a thioesterase (TE) releasing domain and synthesize single ring aromatic polyketides. Groups II, III, and IV contain a thioesterase/Claisen cyclase (TE/CLC) releasing domain and synthesize multiple ring aromatic polyketides. Group V NR-PKSs do not contain a releasing domain and the aromatic polyketide is released by a β-lactamase-type TE (β-TE) encoded by a separate gene. Group VI and VII are CMeT containing NR-PKSs. They synthesize single ring benzoic acid and benzaldehyde after release by the TE (group VI) or reductive (R, group VII) domain, respectively. When an HR-PKS gene or fatty acid synthase (FAS) genes are

near the NR-PKS, the HR-PKS or FAS are likely to synthesize the starter unit for the NR-PKS. Understanding the priming and releasing mechanisms of NR-PKSs gave us some confidence that we could predict what genes might need to be co-expressed to obtain the released NR-PKS products. We have analyzed the *A. terreus* NR-PKSs and placed them into these groups (Figure 4).

We transferred each *A. terreus* NR-PKS gene into *A. nidulans* and confirmed that the transfers were correct by diagnostic PCR (Supplementary Figure S1). Based on our previous results and analyses,[43] we expected that expression of five of the NR-PKSs would result in product synthesis and release without co-expression of additional genes. Induction of expression of ATEG_00145.1, ATEG_03629.1 and ATEG_03432.1 resulted in the production of metabolites detected by liquid chromatography mass spectrometry (LC/MS) as predicted (Figure 5). [Note: all *A. terreus* gene designations are from the Broad Institute *Aspergillus* Comparative Database. *Aspergillus nidulans* genes from this database (below) have the prefix ANID. *A. nidulans* genes from the *Aspergillus* genome database (AspGD, www.aspgd.org) (below) have the prefix AN.] From large-scale cultures, we isolated the products and determined their structures by spectroscopic methods. ATEG_00145.1 produced compounds **1** – **4**, ATEG_03629.1 produced compound **5**, and ATEG_03432.1 produced compounds **6** and **7** (Figure 4a, e, and g). Compounds **6** and **7** are new to science.

Products were not detected for ATEG_10080.1 and ATEG_07500.1. Comparisons of ATEG_10080.1 with the highly homologous *A. nidulans* gene ANID_08383.1 suggested that the correct start codon might be upstream of the annotated one (Supplementary Figure S4). We made a new construct using the alternative start codon and transferred it into *A. nidulans*. Induction of the new construct resulted in the production of a major compound **8** (Figure 4f and 5). Reannotation and expression of this gene in *Aspergillus oryzae* was recently reported by another group.[44]

We revised the start codon for ATEG_07500.1 based on its identity/similarity (93%/96%) to an unpublished *A. terreus* naphthopyrone synthase (GenBank accession no. BAB88689.1) cloned by Watanabe and Ebizuka and transferred a construct with the new start codon into *A. nidulans*. However, we were still unable to detect new products upon induction (Figure 5).

ATEG_08451.1 and ATEG_02434.1 fall into our group V and lack TE domains. For such PKSs, β-lactamases encoded by other genes provide the activity required to release their products.[45–47] The PKS encoded by ATEG_08451.1 (which has been designated ACAS) has been shown to produce atrochrysone (**9**) and its derivatives when co-incubated *in vitro* with ACTE, a β-lactamase encoded by ATEG_08450.1.[46] We transferred ATEG_08451.1 and ATEG_08450.1 into *A. nidulans* and upon induction, atrochrysone (**9**) and its derivative emodin (**10**) were readily detectable by HPLC-DAD-MS (Figure 4c, 5 and Supplementary Figure S5), confirming previous results.[46]

We identified a putative thioesterase gene (ATEG_02438.1) near ATEG_02434.1 that might be responsible for product release. We also deduced that the start codon of ATEG_02434.1 was probably incorrectly annotated, by comparing its predicted coding sequence with those of homologous PKS genes from other species of *Aspergillus*. We transferred ATEG_02438.1 and the reannotated ATEG_02434.1 into *A. nidulans*. Induction of both genes resulted in production of several metabolites (Figure 5). However, we were unable to identify these metabolites due to their instability. Although we have not yet attempted to transfer additional genes, such transfers might result in products that are more stable and amenable to analysis.

Our analyses predicted that the two group VII NR-PKSs, ATEG_07661.1 and ATEG_08662.1, would use precursors produced by an adjacent HR-PKS and fatty acid synthase respectively. We transferred both ATEG_07661.1 and the adjacent HR-PKS ATEG_07659.1 into *A. nidulans* and upon induction detected compound **11**, an intermediate of the asperfuranone biosynthesis pathway.[48] Similarly, we created an *A. nidulans* strain expressing ATEG_08662.1 and the two fatty acid synthase subunit genes adjacent to it in the *A. terreus* genome, but we could not detect any metabolites upon induction (Figure 5).

In summary, the heterologous expression system we have developed has allowed us to detect compounds produced by all but two *A. terreus* NR-PKSs. In these cases we may not have identified the correct start and stop codons and it is also possible that transfer of additional genes may be required for compound production or release.

Expression of a complete *A. terreus* secondary metabolite biosynthetic pathway in *A. nidulans*

To test the utility of our system we wanted to determine if we could express an entire biosynthetic pathway from *A. terreus* in *A. nidulans*. We selected the biosynthetic pathway containing the NR-PKS ATEG_07659.1 and the HR-PKS ATEG_07661.1. As mentioned, expression of these genes in *A. nidulans* resulted in production of **11**, an intermediate in asperfuranone biosynthesis. The *A. nidulans* asperfuranone (afo) cluster contains seven genes[48] including a transcription factor that regulates expression of the cluster. Our analysis of genes in the vicinity of ATEG_07659.1 and ATEG_07661.1 revealed that they were homologous to the afo genes and that the structure of the cluster mirrored the afo cluster (Figure 6). This suggested that the ATEG_07659.1/ATEG_07661.1 cluster produces asperfuranone or a similar compound.

We deleted the *A. nidulans* afo cluster [AN1029-AN1036 inclusive (=ANID_01029.1-ANID_01036)] by replacing it with *ptrA*, a pyrithiamine resistance gene[49] so that the enzymes of this pathway could not interfere with the analysis of the heterologously expressed cluster. We created strains LO6751 and LO6744 that carry the afo cluster deletion and the transferred ATEG_07659.1 and ATEG_07661.1 genes. We sequentially transferred the remaining four genes of the *A. terreus* cluster into these strains (Figure 6). Induction of five genes resulted in production of asperfuranone (**12**) (Figure 7b, traces ix - xi). The expression of a sixth gene, a putative pump homolog, did not alter asperfuranone production (Figure 7b, trace xii). These genes thus constitute an asperfuranone biosynthetic cluster in *A. terreus*. We have cultured NIH2624, the sequenced strain from which we transferred the genes, under various conditions and have not identified asperfuranone. We have, thus, been able to express a pathway that is normally silent in *A. terreus* and identify it as the asperfuranone biosynthetic pathway. The *A. nidulans* asperfuranone cluster is normally silent as well, and to obtain expression we previously had to replace the promoter of a transcription factor gene (*afoA*) associated with the cluster with *alcA*(p) and induce its expression. The induced expression of *afoA* resulted in expression of the entire cluster and production of asperfuranone.[48] We have given the genes of the *A. terreus* pathway designations (Figure 6) based on homology to the *A. nidulans* genes.[48] Thus, for example, the *A. terreus* homolog of the *A. nidulans afoA* gene is designated *AteafoA* following the convention: (http://www.aspergillus.org.uk/indexhome.htm?secure/sequence_info/nomenclature.htm~main) and the product of this gene is AteAfoA.

We obtained a yield of 6.87 ± 0.85 mg/L of asperfuranone in the heterologous expression strain, slightly more than we obtained in parallel experiments with the endogenous *A. nidulans* afo cluster with *alcA*(p)*afoA* induced (5.63 ± 0.33 mg/L). Our heterologous expression system, thus, gives good yields of asperfuranone and we anticipate that this will be the case with compounds produced by other heterologously expressed clusters. We noted,

however, that significant quantities of intermediates were present in the induced heterologous expression strain. Our interpretation is that in the endogenous pathway, the various genes are expressed at optimal levels to result in a single final product, whereas in our system all genes are expressed at high levels resulting in accumulation of intermediates.

Expression of combinations of *Ateafo* genes allows refinement of the asperfuranone biosynthesis pathway

Finally, we have expressed various combinations of *A. terreus* asperfuranone cluster genes that have caused us to revise our proposed biosynthetic pathway for asperfuranone.[48] The *A. nidulans* afo cluster was deleted in all of these strains so that the genes of this cluster could not affect our heterologous expression results. Results are shown in Figure 7. As mentioned, expression of *AteafoG* and *AteafoE* resulted in the production of the polyketide intermediate compound **11** (Figure 7b, traces i and ii). Since, at the onset, the order of the next three genes in the pathway was unclear, we transferred each of them separately into an *AteafoG/ AteafoE* expression strain. Expression of *AteafoF* in the *AteafoG/AteafoE* expression strain did not result in a new product, indicating that AteAfoF does not modify **11** (Figure 7b, trace iii). Compounds **13** and **14** are spontaneously cyclized products of **11** when it is dissolved in methanol during LC-MS analysis (Figure 8a).[48] Expression of *AteafoD* in the *AteafoG/ AteafoE* expression strain resulted in the production of **16** and **17** and the disappearance of **11** suggesting that AteAfoD is the next enzyme in the pathway (Figure 7b, trace iv). The structures of **16** and **17** both contain a hydroxyl group at C-4 (Figure 8b, for details of structural elucidation, see supplemental data). This suggested that the function of AteAfoD is dearomatization of the precursor **11** to generate intermediate A (Figure 8a). Compound **16** could be produced from intermediate A after cyclization and dehydration (Figure 8b). The cyclization step is likely to be spontaneous due to the formation of the six-membered ring hemiacetal intermediate. Moreover, the dearomatization of **11** increases the electrophilicity of the C-1 aldehyde which could facilitate the cyclization. Compound **17** could be generated from **16** by endogenous reductases or from intermediate A via cyclization, dehydration, and reduction mechanisms after reduction of the C-1 aldehyde to a primary alcohol by an unknown endogenous reductase (Figure 8b).

An unexpected result was that addition of *AteafoC* into the *AteafoG* and *AteafoE* expression strain resulted in increased yields of compounds **11, 13**, and **14** (Figure 7b, traces ii and v). This suggested that AteAfoC was involved in an early step of **11** biosynthesis. Although AteAfoC is homologous to the putative oxidoreductase CtnB,[48,25] AteAfoC does contain an esterase/lipase domain. Thus, AteAfoC might be involved in the releasing of the side chain **15** from AteAfoG or might facilitate the side chain transfer from AteAfoG to AteAfoE. To test this hypothesis we created an *AteafoG* and *AteafoC* co-expression strain. Co-expression of *AteafoG* and *AteafoC* resulted in the production of the side chain **15** albeit at a very low level (~1 mg/L, Figure 7b, trace vi). Considering the high yield of **11** (~300 mg/L) in the AteAfoG, E, and C coexpression strain, the low yield of **15** might indicate that the function of AteAfoC is to facilitate the transfer of **15** from AteAfoG to AteAfoE.

As expected, adding *AteafoD* to the *AteafoG, AteafoE* and *AteafoC* expression strain resulted in increased yields of intermediates **16** and **17** in comparison with the *AteafoG, AteafoE*, and *AteafoD* expressing strain (Figure 7b, traces iv and vii). This led us to test whether we could further increase the yield by expressing two copies of *AteafoC*. Indeed, the yield of **16** and **17** in the strain with two copies of *AteafoC* was about 1.5 fold higher than in the strain that contained only one copy of *AteafoC* (Figure 6B, traces vii and viii). We were able to purify minor metabolites **18** and **19**, which are shunt products, from the strain expressing two copies of *AteafoC*. Compounds **17, 18** and **19** are new to science. This

highlights an advantage of our marker recycling system, that we are able to increase the metabolite yield simply by increasing the gene copy number of rate limiting enzymes.

By adding *AteafoF* into the *AteafoG*, *AteafoE*, *AteafoC* and *AteafoD* expression strain, as mentioned, we successfully reconstituted the entire AteAfo pathway in *A. nidulans*, to produce asperfuranone (**12**, Figure 7b, trace ix) (in effect assembling our own asperfuranone operon). Thus, AfeAfoF functions as a C-8 oxygenase to generate **20**. Introducing the hydroxyl group at C-8 prevented the C-8, C-9 enol formation, thus, suppressing the formation of a six-membered ring hemiacetal intermediate. After cyclization and dehydration of **20** via a five-membered ring hemiacetal intermediate, **21** could be generated (Figure 8a). Since we did not add a reductase into the asperfuranone (**12**) producing strain, the conversion from **21** to **12** is likely to be catalyzed by an endogenous reductase. The afo and Ateafo clusters do contain a putative dehydrogenase or reductase (AN1030 and ATEG_07665.1). The fact that deletion of AN1030 did not alter the yield of asperfuranone[48] suggested that, if this gene is indeed involved in the transformation from **21** to **12**, the function can be compensated by an unknown endogenous reductase encoded by a gene somewhere else in the genome.

To see if the position of the genes in the newly constructed pathway was important, we created two additional strains in which the order of *AteafoC*, *AteafoD* and *AtoafoF* were different, making a total of three strains in which the order of these genes was different. All three strains produced nearly identical metabolite profiles (Figure 7b, traces ix - xi), suggesting that position effects are minimal or non-existent and that we can sequentially integrate all putative biosynthetic genes from an unknown gene cluster into our host without considering the location of each gene. It is interesting to note that, as mentioned, the HPLC profile did not change obviously after coexpression of the putative efflux pump *AteafoB* with the *AteafoC-G* expression strain (Figure 7b, traces x and xii). Thus, the function of AteAfoB or its homolog AfoB is unlikely to be a transporter of the identified metabolites in the afo biosynthetic pathway. Deletion of *afoB* resulted in a decreased production of asperfuranone (**12**),[48] however, so AfoB does play some role in asperfuranone production in *A. nidulans* when the afo pathway genes are under control of their normal promoters. This raises the possibility that AfoB has a function in regulation of the pathway, but the function of AfoB remains elusive and awaits further study.

From results of our reconstitution study, our original hypothesis, that the function of AfoD is to oxidize the C-8 benzylic carbon of **11** to become intermediate B, needs to be revised (Figure 8A).[48] Davison et al.[50] and Zabala et al.[51] recently reported that the function of the AfoD homologs, TroB from *Talaromyces stipitatus* and AzaE from *Aspergillus niger* is dearomatization of the aromatic product released from an NR-PKS. Our data support and confirm their results. Moreover, Zabala et al. speculated that AfoC might facilitate the transfer of **15** from AfoG to AfoE.[51] *In vivo* evidence from our heterologous expression studies support their hypothesis.

Prospects for expressing additional SM genes

Our system for expressing SM genes from other fungi in *A. nidulans* is rapid, robust and flexible. It has allowed us to express and purify the products of six NR-PKS genes of *A. terreus*, to identify two compounds new to science and to assemble and express an entire *A. terreus* biosynthetic pathway, which resulted in the identification of the *A. terreus* asperfuranone biosynthetic cluster, a previously cryptic cluster. Importantly, our approach bypasses normal regulatory mechanisms. Arguably, we understand SM cluster regulation better in *A. nidulans* than in any other fungus, but we still do not understand how to activate most SM clusters.[43] While we might be able to transfer intact clusters from other fungi into *A. nidulans* by other mechanisms, this would be only a starting point for obtaining

expression. In our system, by placing each gene under control of *alcA*(p), we control expression. Glucose represses *alcA*(p) and if transferred genes produce compounds toxic to *A. nidulans* they will not be produced as long as glucose is present so transformants will survive. We can then induce *alcA*(p) and produce the toxic compounds, at least transiently. The *alcA* promoter can drive gene expression at high levels,[30, 52] resulting in good product yields. For very large clusters, AlcR, the activator of *alcA*(p), might be limiting, but this can be remedied by expressing *alcR* from a high level constitutive promoter.[53] In principle, other promoters can also be used with our approach.

Our approach is most obviously useful for expressing and identifying the products released by NR-PKS from diverse species of *Aspergillus* and expressing relatively small SM gene clusters or portions of larger clusters. There is no obvious technical barrier to transferring all the genes of large clusters into *A. nidulans*, however, and our approach is quite rapid. Transferral of a gene and verification takes about two weeks and transferral of genes of many clusters can be carried out in parallel. However, as the number of transferred genes increases the likelihood of failed expression due to incorrect annotation increases. This limitation will be reduced as annotation procedures improve. Our approach requires very little DNA and if an accurate sequence can be obtained and annotated correctly, it can, in theory, be even used to express SM clusters from fungi that are currently not culturable.

In principle, our approach should work for clusters containing HR-PKS, NRPSs and terpene synthases or cyclases and we are in the process of testing the efficacy of this approach for these enzymes. It is also likely that *A. nidulans* can be used as a heterologous host for expression of SM genes from genera of fungi other than *Aspergillus*. However, given the fact that there are approximately 250 known species of *Aspergillus*[54] and each species likely has roughly 10 NR-PKSs, even if our approach works only for relatively small NR-PKS clusters in *Aspergillus spp.* it has the potential to unlock a substantial storehouse of natural products.

Conclusion

We have developed an efficient system for heterologous expression of fungal secondary metabolism genes in *Aspergillus nidulans*. This system includes methods for deletion of entire *A. nidulans* secondary metabolite gene clusters to eliminate production of the most abundant *A. nidulans* SMs including important toxins. This reduces the SM background and facilitates detection and purification of the products of heterologously-expressed clusters. It also includes a method for amplifying secondary metabolism genes from other fungi, placing them under the control of a regulatable promoter and inserting the genes at a target locus in *A. nidulans*. Our system incorporates selectable marker recycling so that an unlimited number of genes can be transferred into, and expressed in, *A. nidulans*. We have used this system to obtain compound production and release from six NR-PKSs of *A. terreus* and we have identified the products. We have transferred and expressed all the genes of a cryptic cluster from *A. terreus* and have found that the cluster produces asperfuranone. In addition, by expressing the genes in the asperfuranone cluster in various combinations and purifying and analyzing the compounds produced, we have clarified the asperfuranone biosynthetic pathway.

Experimental Section

Strains

Strains used in this study are listed in Supplementary Tables S1 and S2.

Molecular genetic manipulations

Transforming fragments were generated by fusion PCR essentially as described.[34, 55, 56] To reduce the frequency of PCR-induced mutations, we used high-fidelity DNA polymerases. For fragments < 2kb, we generally used AccuPrime Pfx DNA polymerase (Invitrogen). For larger fragments we used KOD DNA polymerase (EMD Biosciences) or Phusion DNA polymerase (New England Biolabs or Thermo) and we used these enzymes for fusion PCR as well. Ramp rates were as previously described,[34, 55, 56] but for AccuPrime Pfx the annealing temperature was T_m (for the lowest melting temperature primer) − 5°C (T_m calculated as previously described[55]). For KOD and Phusion DNA polymerases the annealing temperature was $T_m + 2°C$ and the extension rate was 30 sec/kilobase of expected product. Similarly, the constructs used for cluster deletions were made by fusion PCR with AccuPrime Pfx DNA polymerase used for amplification of fragments < 2kb and KOD or Phusion DNA polymerases used for larger fragments and for fusion PCR.

Production of protoplasts and transformations were carried out as described[34, 55, 56] except that, because of the discontinuance of production of VinoFlow FCE, in later experiments VinoTaste Pro (Novo) was used at a final concentration of 100 mg/ml for protoplasting.[56] For selection of transformants on 5-FOA, 1 mg/ml 5-FOA, 10 mM uridine and 1mg/ml uracil were added to selection plates. Selection for cluster loop outs on 5-FOA was as previously described.[37]

Diagnostic PCR was carried out as described[57] using Platinum Taq DNA polymerase (Invitrogen) or OneTaq Hot Start Quickload (New England Biolabs). Diagnostic PCR strategies are shown in Supplemental Figure S1.

Fermentation and LC/MS analysis

For *alcA*(p) induction, 3×10^7 spores were grown in 30 ml liquid LMM medium (15 g/l lactose, 6g/l $NaNO_3$, 0.52g/l KCl, 0.52g/l $MgSO_4$ $7H_2O$, 1.52g/l KH_2PO_4, 1 ml/l trace elements solution) in 125 ml flasks at 37°C with shaking at 180 rpm and supplemented with riboflavin (2.5 mg/l), or pyridoxine (0.5 mg/l) when necessary. Cyclopentanone was added to the medium at a final concentration of 10 mM 18 hr after inoculation. Culture medium was collected 48 hr after cyclopentanone induction by filtration and extracted with the same volume of EtOAc. In order to extract most acidic phenolic compounds, the water layer was extracted with the same volume of EtOAc after acidification (pH = 2). EtOAc extract was then evaporated by TurboVap LV (Caliper LifeSciences). The crude extracts were re-dissolved in 0.5 ml of DMSO:MeOH (1:4) and 10 μl was injected for LC-DAD-MS analysis as described previously.[58] Details for large-scale purifications of compounds for each strain and compound identification are given in the Supplemental Information as are spectral data.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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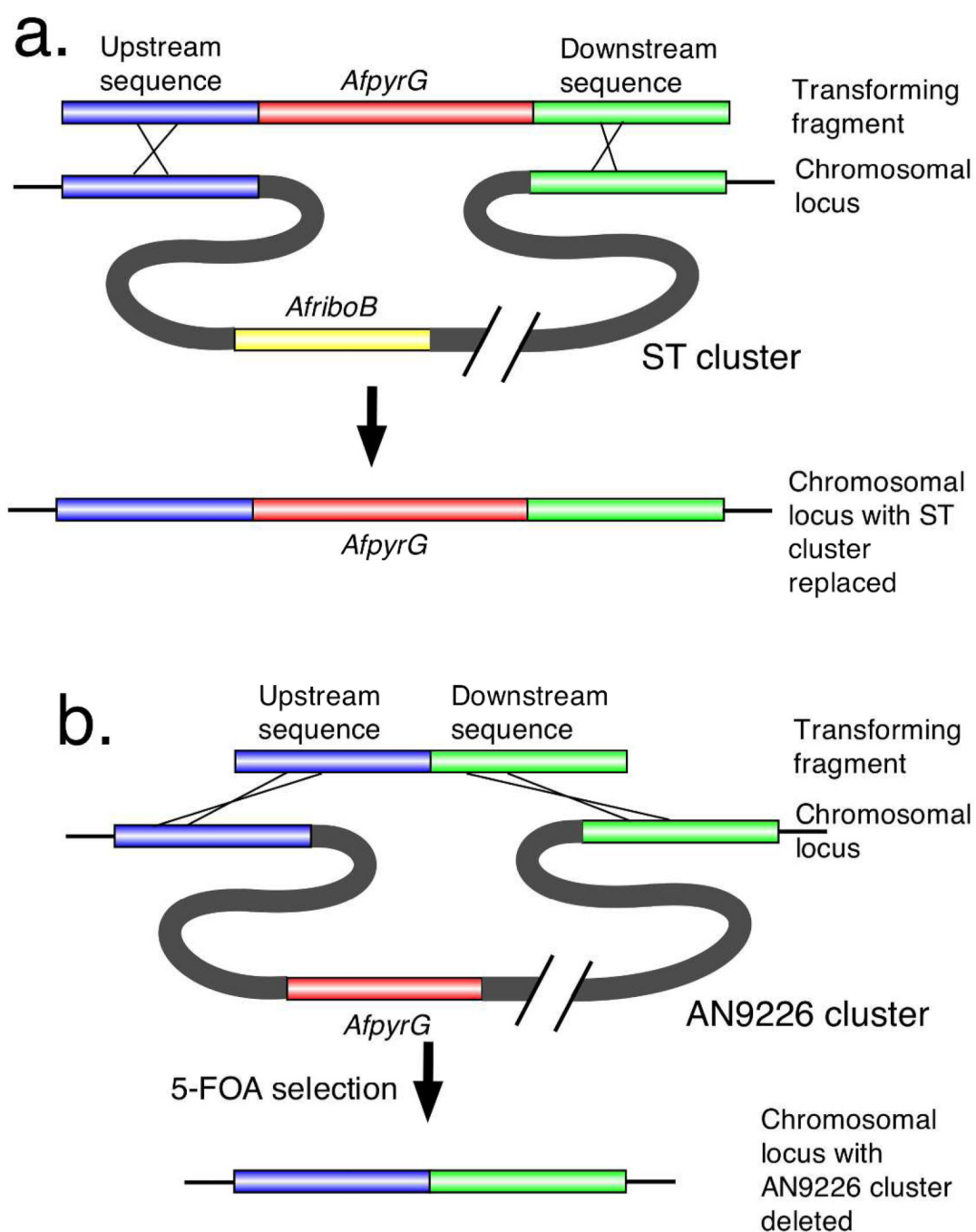
Figure 1.
Two strategies for deletion of SM gene clusters. a. The target strain is transformed with a fragment containing the *A. fumigatus pyrG* gene (*AfpyrG*) flanked by two 2 kb sequences that are > 50 kb apart in the genome, one upstream of the ST cluster and one downstream. The fragment is constructed by fusion PCR[34,55] and the flanking fragments are amplified from genomic DNA using primers designed from genome sequence data. Upon transformation, homologous recombination results in the replacement of the entire ST cluster with *AfpyrG*. *AfriboB* was previously inserted in the cluster and replacement of the cluster with *AfpyrG* results in removal of *AfriboB* and this causes riboflavin auxotrophy. b. The *AfpyrG* gene was previously inserted into the target cluster by transformation. The

transforming fragment is composed of two sequences, one from just upstream of the cluster and the other from just downstream of the cluster. They are amplified from genomic DNA and joined by fusion PCR. Upon transformation, homologous recombination results in excision of the target cluster including *AfpyrG*. Transformants in which *AfpyrG* has been excised are selected on 5-FOA. *AfpyrG* can be subsequently reused as a selectable marker.
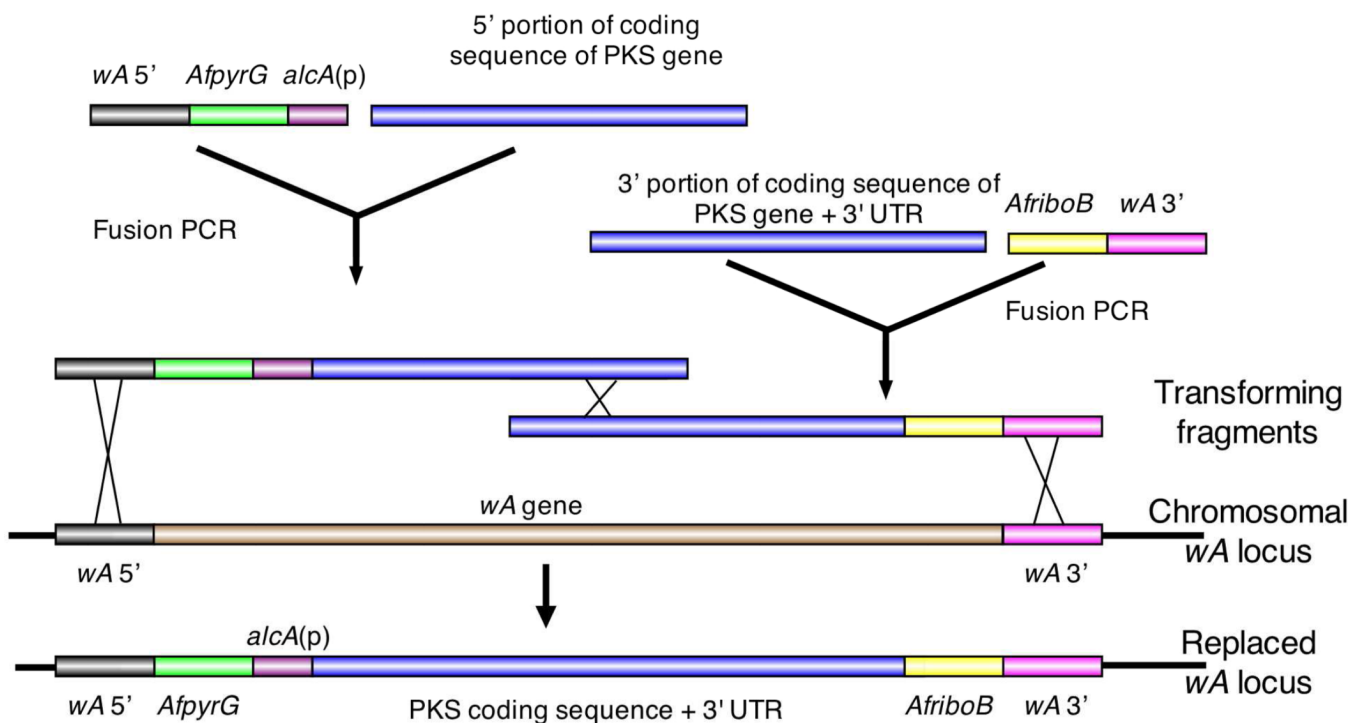
Figure 2.
Transferral of a PKS gene into *A. nidulans*. A portion of a PKS gene extending from the start codon is amplified from genomic DNA of the target organism and joined by fusion PCR to a cassette containing ≈ 1000 bp of flanking sequence upstream of the *A. nidulans* *wA* (white conidia) gene, *AfpyrG* and *alcA*(p). A 3' portion of the target PKS gene overlapping by approximately 1 kb with the 5' portion and extending approximately 100 bp downstream of the termination codon is fused to a cassette containing *AfriboB*[34] and approximately 1000 *bp wA* downstream flanking sequence. The fusion PCR products are co-transformed into the target strain, LO4389. LO4389 has green conidia and carries *nkuA*Δ, a deletion of the ST cluster and three selectable markers, [*pyrG89* (pyrimidine auxotrophy), *riboB*2 (riboflavin auxotrophy), and *pyroA*4 (pyridoxine auxotrophy)]. Upon transformation the two fragments undergo homologous recombination with the *wA* flanking sequences and with each other, resulting in replacement of the *wA* gene with a full-length coding sequence of the PKS under control of *alcA*(p). Transformants are selected for both pyrimidine and riboflavin prototrophy and correct transformants have white conidia. The procedure works equally well for NRPS and other large genes.
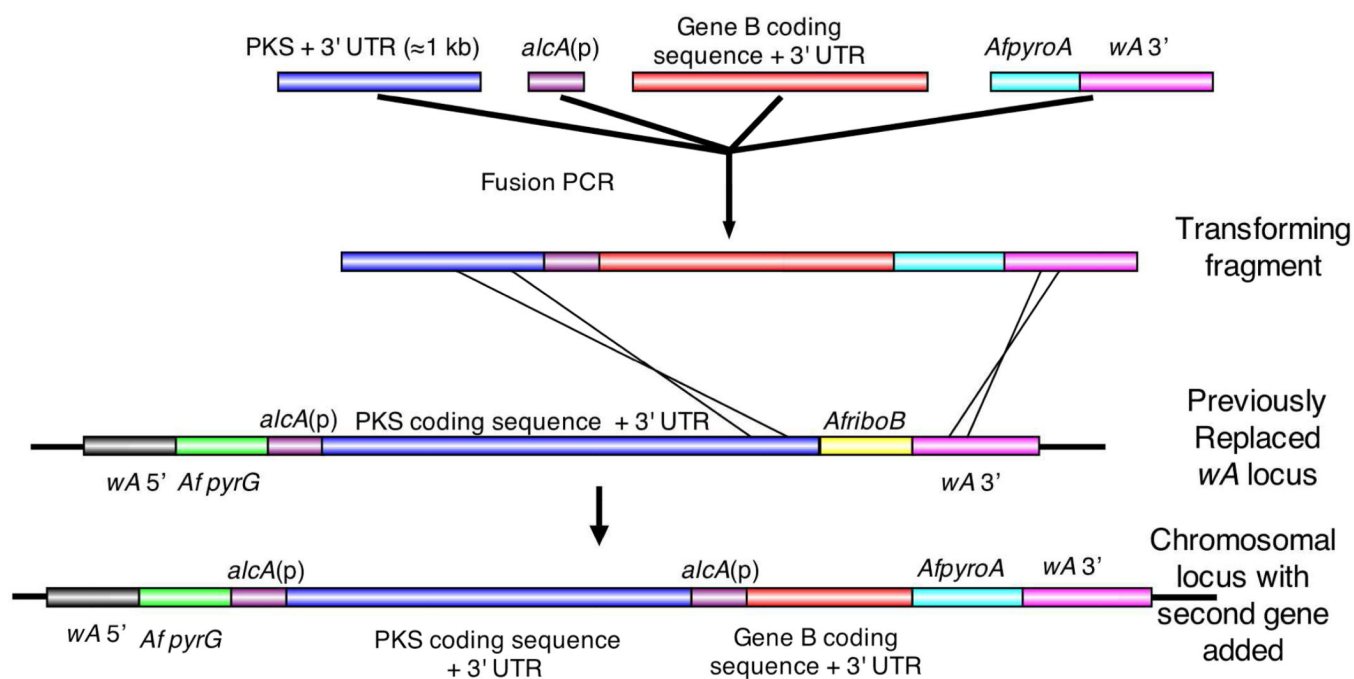
Figure 3.
Transferral of additional genes with marker recycling. Using four piece fusion PCR, we create a transforming fragment containing approximately 1000 bp from the C-terminus of the PKS already inserted (approximately 900 bp of coding sequence plus 100 bp of 3' untranslated region), *alcA*(p), the coding sequence of the gene of interest plus 100 bp of 3' untranslated region, *AfpyroA*, a selectable marker[34], and 1000 bp of *wA* downstream sequence. Transformation with this fragment inserts the gene, under the control of *alcA*(p), and it evicts *AfriboB* allowing it to be used as a selectable marker for the next transformation. In principle, recombination between *alcA*(p) on the transforming fragment and *alcA*(p) upstream of the PKS could cause deletion of the PKS, but in practice this event did not occur frequently enough to hinder progress. To add a third gene the process shown in b is repeated except that *AfriboB* is used as a selectable marker and *AfpyroA* is excised upon transformation. The process can be repeated over and over.
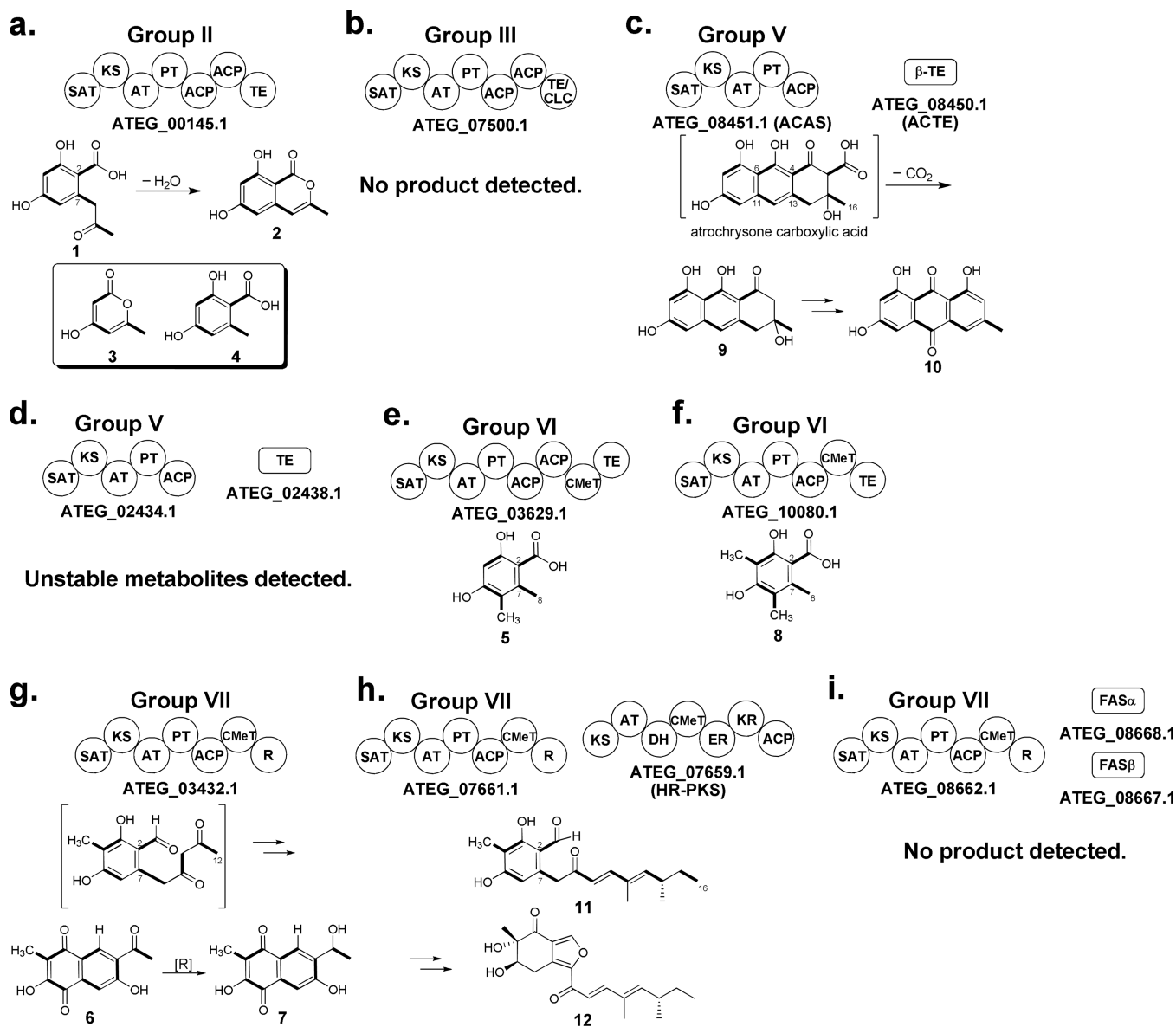
Figure 4.
Aromatic polyketides identified from heterologous expression of *A. terreus* NR-PKSs together with accessory genes in the engineered *A. nidulans* host. The domain structure of each NR-PKS is shown as well as the product released. Defining features of the groups are discussed in.[43]
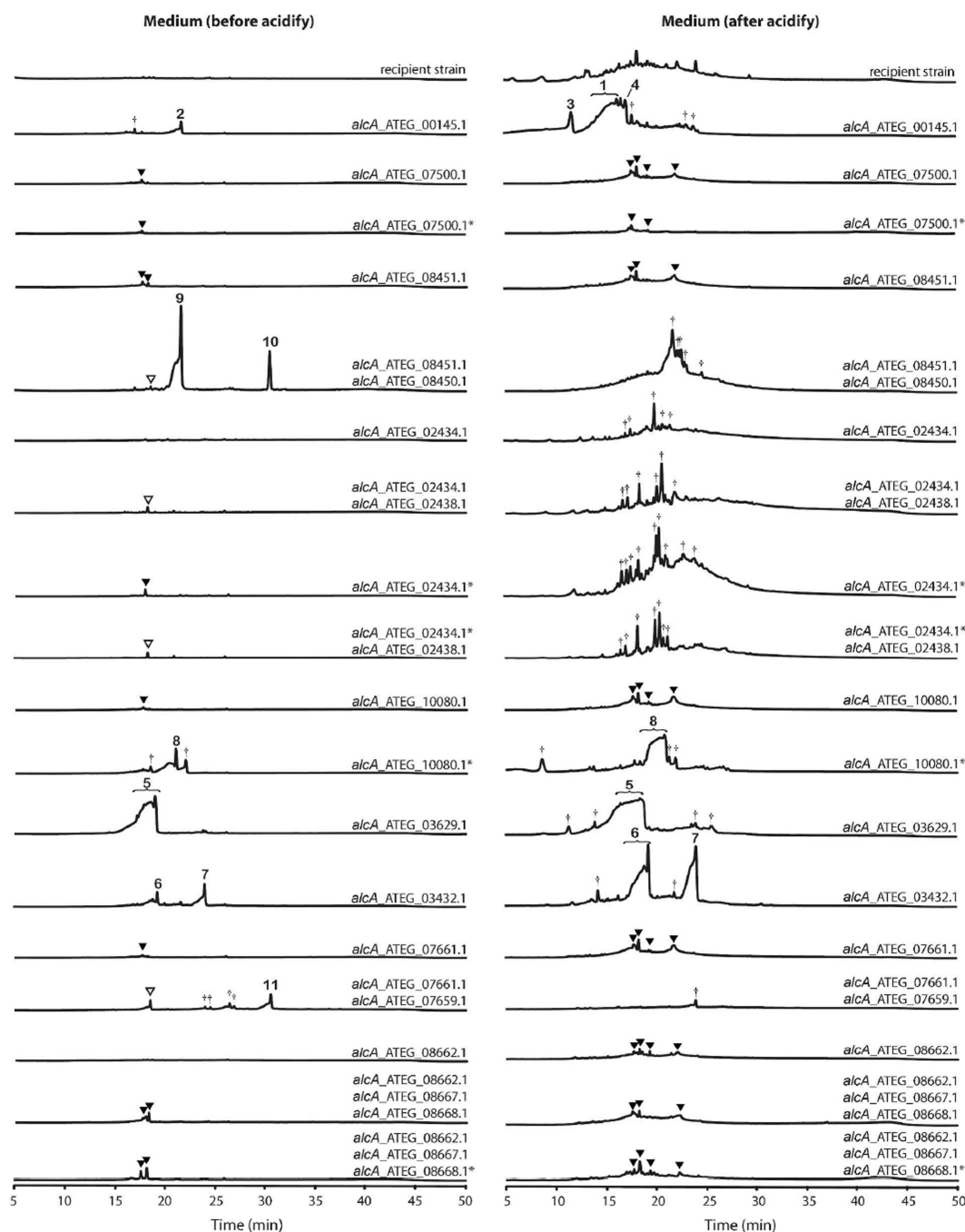
Figure 5.
Total scan HPLC profiles of metabolites extracted from the culture medium before or after acidification of *A. nidulans* strains expressing *A. terreus* genes under the control of *alcA*(p). The genes expressed are listed above each trace. The numbering of the peaks corresponds to compounds shown in Figure 4. Peaks that are also present in other strains are marked by a black triangle (▼) and are considered not to be specific to the expressed genes. Peaks that are specifically present in induced strains but are not structurally elucidated due to low yield or pool stability are marked with a dagger (†). ∇: Lumichrome, a degraded product from riboflavin. *: Transferred genes with a revised start site.
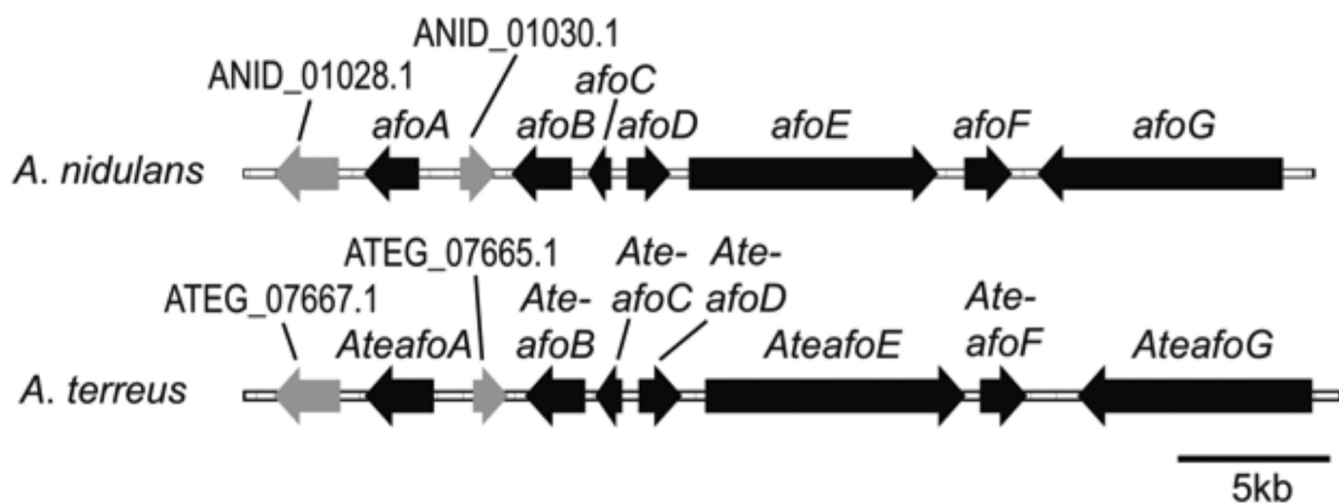
A. nidulans	A. terreus	Putative function and identity/similarity (%)
ANID_01028.1	ATEG_07667.1	regulator (63/72)
ANID_01029.1 (afoA)	ATEG_07666.1 (AteafoA)	positive regulator (61/71)
ANID_01030.1	ATEG_07665.1	dehydrogenase (81/88)
ANID_01031.1 (afoB)	ATEG_07664.1 (AteafoB)	efflux pump (76/82)
ANID_01032.1 (afoC)	ATEG_07663.1 (AteafoC)	esterase/lipase (57/66)
ANID_01033.1 (afoD)	ATEG_07662.1 (AteafoD)	salicylate monooxygenase (70/84)
ANID_01034.1 (afoE)	ATEG_07661.1 (AteafoE)	NR-PKS (76/85)
ANID_01035.1 (afoF)	ATEG_07660.1 (AteafoF)	FAD-dependent oxygenase (73/83)
ANID_01036.1 (afoG)	ATEG_07659.1 (AteafoG)	HR-PKS (76/85)

Figure 6.
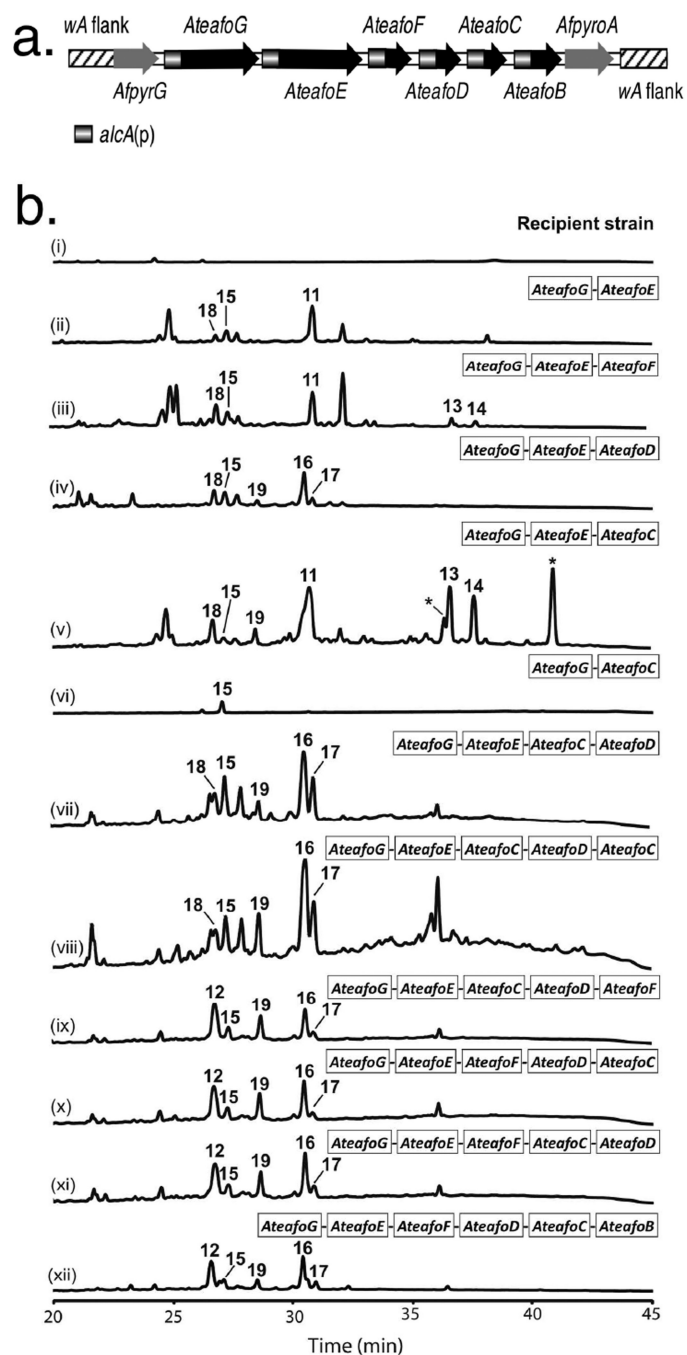Gene organization of the *A. nidulans* afo and *A terreus* Ateafo clusters.

Figure 7.
The *A. terreus* asperfuranone (Ateafo) gene cluster assembled in *A. nidulans* with each gene placed under control of the *alcA* promoter. Total scan HPLC profiles of metabolites extracted from the culture media of *A. nidulans* strains expressing *A. terreus* afo cluster genes under the control of *alcA*(p). The numbering of the peaks corresponds to compounds shown in Figure 8. The *Ateafo* genes expressed are listed above each trace. (i) is the trace for the control strain. *Unstable metabolites that have different chromophores from those compounds identified in this study.
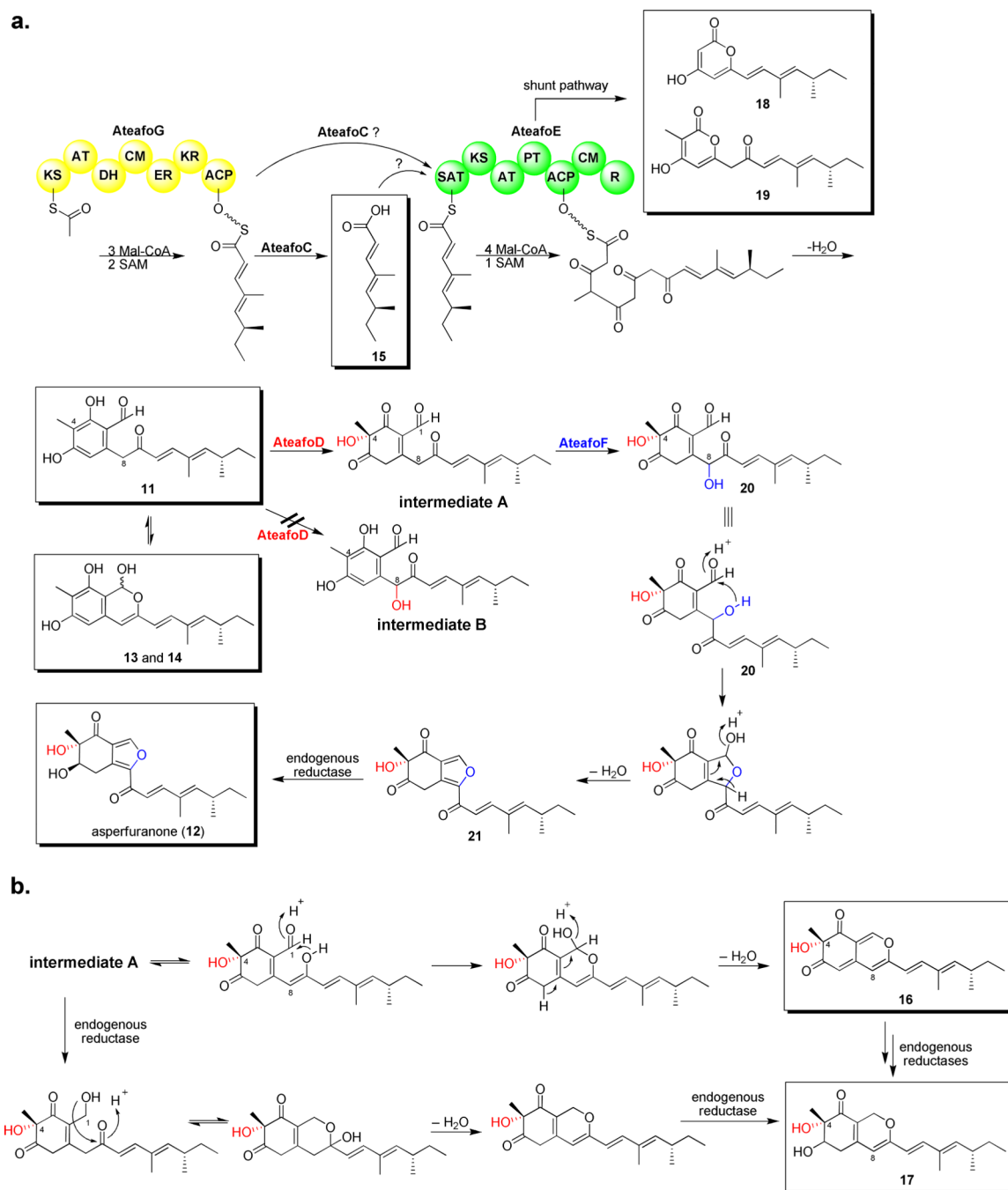
Figure 8.
a. A revised biosynthetic pathway of asperfuranone from reconstitution studies. b. Proposed biotransformation from intermediate A to **16** and **17**.

Table 1

Promoters and selectable genes used in this study.

	Description	Reference(s)
alcA(p)	The promoter from the *A. nidulans alcA* (alcohol dehydrogenase) gene. The promoter is strongly repressed by glucose and induced to various levels by alcohols and other compounds.	30, 31
AfpyrG	The *Aspergillus fumigatus* homolog of the *A. nidulans pyrG* gene. *pyrG* encodes orotidine-5'-phosphate decarboxylase. An *A. nidulans pyrG* mutation (*pyrG*89) renders the enzyme dysfunctional and causes a requirement for uridine or uracil. *AfpyrG* complements *pyrG89* restoring growth in the absence of uridine or uracil.	33
AfriboB	The *A. fumigatus* homolog of the *A. nidulans riboB* gene. *riboB2* is a mutation that causes a requirement for riboflavin. *AfriboB* complements *riboB*2, restoring growth in the absence of riboflavin.	34
AfpyroA	*AfpyroA* is the *A. fumigatus* homolog of the *A. nidulans pyroA* gene. *pyroA4* is a mutation that causes a requirement for pyridoxine. *AfpyroA* compements *pyroA*4, restoring growth in the absence of pyridoxine.	34