Letter

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Rewiring of the austinoid biosynthetic pathway in filamentous fungi

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Abbreviations: NP, natural product; PKS, polyketide synthase

Running title: Genome-based rewiring of meroterpenoids

Key words: *Aspergillus*, *Penicillium*, meroterpenoids, austinoids, genome mining, natural products

Abstract

Filamentous fungi produce numerous high-value natural products (NPs). The biosynthetic genes for NPs are normally clustered in the genome. A valuable NP class is represented by the insecticidal austinoids. We previously determined their biosynthesis in the fungus *Aspergillus calidoustus*. After further computational analysis looking into the austinoid gene clusters in two additional distantly related fungi, *Aspergillus nidulans* and *Penicillium brasilianum*, a rearrangement of the genes was observed which corresponded to the diverse austinoid derivatives produced by each strain. By advanced targeted combinatorial engineering using polycistronic expression of selected genes, we rewired the austinoid pathway in the fungus *A. nidulans*, which then produced certain compounds of interest under industrially favored conditions. This was possible by exploiting the presence of previously thought irrelevant genes. Our work shows that comparative analysis of genomes can be used not only to discover new gene clusters, but unearth the hidden potential of known metabolic pathways.

Natural products (NPs) produced by microorganisms are invaluable sources for bioactive compounds.[1, 2] Typically these NPs are biosynthesized by genes that are clustered in the genome and often possess multimodular enzymes, such as polyketide synthases (PKSs) and/or nonribosomal peptide synthetases (NRPSs).[3] Further chemical modifications of polyketide or nonribosomal peptide products are catalyzed by tailoring enzymes that can change the backbone of these molecules.[1] Some polyketides can be further modified by prenyltransferase enzymes resulting in products belonging to the meroterpenoid compound class.[4]

Meroterpenoids have been identified in various filamentous fungi such as anditomin from *Aspergillus variecolor*, terretonin from *Aspergillus terreus*, andrastins from *Penicillium chrysogenum*, and paraherquonin from *Penicillium brasilianum*.[5-8] Interestingly, different meroterpenoids have been shown to possess selective insecticidal activity.[9] For example, among the austinoids, derivatives have been found to even exert a species-specific activity towards different insects.[10]

The austinoids were first isolated in 1976 from *Aspergillus calidoustus* (previously assigned as *Aspergillus ustus*)[10, 11], and later identified in both *A. nidulans*[12, 13] and *P. brasilianum*.[5] Recently, a study involving the elucidation of the most complex austinoid gene cluster in *A. calidoustus* revealed that a second PKS, a noniterative diketide synthase, is also taking part in the biosynthesis.[10] In the present study, we set out to obtain further insight on the genomic arrangement of the austinoid gene clusters and look into the chemotypes within these producing strains. Furthermore, we applied advanced synthetic biology techniques to produce various austinoid derivatives in different growth stages in the model organism *A. nidulans*.

The work presented here includes an intriguing example of a gene cluster's rearrangement among three distantly related fungal species. These species not only produce different austinoid derivatives,

but they accumulate these molecules during different growth stages. Using comparative genomics and heterologous expression, we could reconstitute the entire austinoid pathway in *A. nidulans* rewiring the complete production of these potentially valuable compounds.

The three known austinoid producing species, *A. calidoustus*, *A. nidulans* and *P. brasilianum*, have had their genomes sequenced[14-16] and it was previously shown that *A. nidulans* produces the least number of austionoid derivatives with the end products austinol **1** and dehydroaustinol **8**[17], while *P. brasilianum* can produce until acetoxydehydroaustin **10**[18], and *A. calidoustus* produces the highest number of identified derivatives (Figure 1).[10] Comparative genome analysis confirmed that the austinoid chemical variability was also accompanied by a genetic fluctuation between the different gene clusters (Figure 2 and Table S1). In *A. calidoustus*, the austinoid gene cluster lies on a 66 kb contiguous DNA region, while clusters from *A. nidulans* and *P. brasilianum* are split in their respective genomes. As shown, the genetic rearrangement provoked certain variability among the clusters. In particular, several *A. calidoustus* genes are absent in the other two species, namely *ausO*, *ausT*, *ausV* and *ausW* (depicted in green in Figure 2). Moreover, in the clusters identified in *A. nidulans* and *P. brasilianum*, the gene order of the loci is different than in *A. calidoustus*, with some genes having different orientations (e.g. *ausE* and *ausK* in *P. brasilianum*). Additionally, *A. calidoustus* is the only species encoding a noninterative diketide synthase in the cluster, *ausV* (Figure 2 and Table 1), while the other two species show only residual pseudogenes having a DNA sequence highly similar to the *ausV* locus (~ 80%, Table S1).

The high nucleotide similarity shared by the clusters of different species, coupled with the presence of the *ausV* pseudogene in *A. nidulans* and *P. brasilianum*, suggests that *A. calidoustus* is the original bearer of the austinoid gene cluster. Indeed, this species produces the highest variety of derivatives that were likely lost in the others when the genetic rearrangements occurred.

Another variation of the austinoid cluster was also suggested in *P. brasilianum*. A genetic rearrangement was hypothesized between two separate *P. brasilianum* strains, NBRC 6234 and MG11. Interestingly, NBRC 6234 strain is unable to produce austinoids, but the closely related compound paraherquonin [5]. Austinoids and paraherquonin share the initial biosynthetic steps, but they differentiate when preaustinoid A is transformed either into preaustinoid A1 or berkeleydione.[5, 12] This biosynthetic step is catalyzed by the non-heme iron-dependent dioxygenase AusE/PrhA.[5, 19] The *in vitro* enzymatic characterization of the purified PrhA from *P. brasilianum* NBRC 6234 revealed that the variation of this single enzyme led to the formation of berkeleydione, an intermediate in paraherquonin biosynthesis.[5]

We decided to exploit the knowledge gained from the comparative analysis of the austinoid gene cluster, with the goal of rewiring the pathway in more favorable fermentation conditions. *A. calidoustus* can produce the most derivatives having insecticide activity, but only during static culture conditions when conidiation occurs (Figure 1B). This condition is unfavorable for industrial scale production as the usual methods involve large fermenters with planktonic growth. Oppositely, *A. nidulans* containing our empty expression vector can produce austinoids during planktonic growth, but only the early derivatives, which did not show any discernable activity (Figure 3A-B). With the goal of creating a metabolically engineered *A. nidulans* strain that produces austinoid derivatives in a more suitable condition, genes from *A. calidoustus* were expressed in *A. nidulans* (Figures S1 and S2). The candidate genes were heterologously expressed under the control of the inducible xylose promoter (*xyl*p) from *Penicillium chrysogenum*. This promoter is induced by xylose and repressed when glucose is the sole carbon source.[20]

The first step was to heterologously express the O-acetyltransferase gene *ausP,* as this gene is not present in the *A. nidulans* cluster. As expected, the LC-MS analysis showed that the resulting

transgenic *A. nidulans* strain was capable of producing both austin **2** and dehydroaustin **3** (Figure 3C). Surprisingly, the strain was also able to further synthesize 7-hydroxydehydroaustin **4** and acetoxydehydroaustin **10** (Figure 3C). This finding showed that the homologues in the *A. nidulans* austinol gene cluster for *ausQ* and *ausR*, which encode for a second O-acyltransferase (AN9250) and a cytochrome P450 monooxygenase (AN9251) respectively, are still fully functional. Even more, they are still being expressed during austinoid production, although irrelevant for the *A. nidulans*-specific biosynthesis (Table S1). Moreover, this result demonstrated that the most active compound, **3**, could be produced during planktonic growth, which is a more industrially favorable condition.

Additionally, three more *A. calidoustus* genes were introduced into *A. nidulans* by targeted combinatorial engineering: the gene coding for the noniterative PKS AusV, the dioxygenase AusO and the progesterone 5-beta-reductase-like protein AusT, all in different combinations with AusP.

Under xylose-inducing conditions, *A. nidulans* strains expressing *ausT* were able to produce 1,2-dihydro-7-hydroxydehydroaustin **5** and shunt pathway derivatives including acetoxydehydroaustin **10** and 1,2-dihydro-acetoxydehydroaustin **11** (Figure 3D). By adding only *ausV*, it was possible to obtain the shunt pathway product leading to 1,2-dehydro-precalidodehydroaustin **13** (Figure 3E).

Further gene combinations of three or more genes were generated by polycistronic expression of selected genes. This method entails the use of 2A viral peptides that separate the individual genes, which when transcribed are cleaved into their individual proteins. This technique is advantageous because of its use of a single inducible promoter, which controls the expression of a polycistronic mRNA in eukaryotes (Figure 3F-G).[21] Interestingly, we could not combine all genes in the same construct because the polycistronic expression of the O-acetyltransferase AusP could not restore production of later derivatives (data not shown). This was likely due to the presence of the 2A tag

on the C-terminus, which likely inhibited its activity. Therefore, further gene additions into *A. nidulans* were transformed into a strain already expressing *ausP*.

The combination of *ausT* and *ausV*, used to transform the *A. nidulans* strain already expressing *ausP*, led to the successful production of precalidodehydroaustin **6** (Figure 3F). Fascinatingly, AusQ from *A. nidulans* could successfully transfer the AusV PKS product to the austinoid scaffold, and the acetyl group as seen in acetoxydehydroaustin **10** and 1,2-dihydro-acetoxydehydroaustin **11**. To finish the reconstitution in *A. nidulans*, a three-gene combination, *ausO*, *ausT* and *ausV*, was assembled on one plasmid and once introduced into *A. nidulans*, it was able to biosynthesize the end product calidodehydroaustin **7** (Figure 3G). Unfortunately, but also of interest, was the fact that austinoids adorned by the AusV diketide product, even if produced in *A. nidulans*, could not be detected in a planktonic culture; hence, static cultures were required for production of compounds precalidodehydroaustin **6**, calidodehydroaustin **7**, and 1,2-dehydro-precalidodehydroaustin **13** shown in Figure 3E, 3F and 3G respectively. Reasons for *A. nidulans* not able to produce the diketide products under planktonic cultures range from the actual AusV enzyme directing the biosynthesis in different growth stages or the chemical modification itself dictates production or even have attachment to the membrane. However, it is important to note that the most active insecticidal derivative, dehydroaustin **3**[10, 22, 23], could be produced under planktonic growth conditions. This could be extremely beneficial in an industrial setting, not only because of the different regulation in comparison to *A. calidoustus*, but also the fact that *A. nidulans* is a model organism in filamentous fungi[24] and if further genetic modifications are needed this fungus is optimal.

This study gives intriguing insights into how gene clusters can be rearranged. This was demonstrated by comparing three different austinoid gene clusters from three diverse filamentous

fungi. Some genes studied here, were analyzed in a previous biosynthetic study appearing to have no apparent function; thus, they were deemed irrelevant. This was the case in *A. nidulans,* where *ausR* and *ausQ* were deleted, but no change was observed.[12] After introduction of *ausP* from *A. calidoustus* into *A. nidulans*, precursors for these enzymes were produced and the deduced products requiring the activity of these redundant enzymes were detected with 7-hydroxydeyhdroaustin **4** and acetoxydehydroaustin **10** (Figure 3C). Thus, we were able to take advantage of these remanent genes and reconstruct the complete austinoid biosynthetic pathway in *A. nidulans* by heterologously expressing the minimal number of remaining genes.

With this study, we highlighted that the knowledge of NPs cannot be merely restricted to the chemical output of the individual fungus. Additional knowledge on genomic and physiological aspects is also important along with available synthetic biology tools that can allow for the production of different active austinoid derivatives under industrially favored conditions. Moreover, dehydroaustin **3**, the most active derivative, could even be synthesized by the model organism *A. nidulans*.

In conclusion, our results demonstrated that the chemical plasticity of NPs might be hidden in similar but not identical clusters of different microbial species. Therefore, comparative genomics could be applied not only to discover new gene clusters, but also to investigate the full potential of previously studied metabolic pathways.

Materials and Methods

Fungal strains and cultivation

Strains used in this study are shown in Table S2, and are deposited in the Jena Microbial Resource Collection (www.uni-jena.de/Pilz__Referenz_Zentrum.html). Two different media were used for fungal growth, *Aspergillus* minimal medium (AMM)[25] and complex medium (1% (w/v) glucose, 0.2% (w/v) peptone, 0.1% (w/v) yeast extract, 0.1% (w/v) casamino acids, 10.8 mM $MgSO_4$, 17.4 mM KCl, 28.7 mM KH_2PO_4, 0.1% (v/v) trace elements, 0.1% (v/v) vitamin solution (stock: 0.07 mM p-aminobenzoic acid, 0.30 mM pyridoxine HCl, 0.008 mM biotin and 0.09 mM pantothenic acid) and 0.33 µM riboflavin. For solid media, agar 1.5% (w/v) was added.

For austinoid production, fungal cultures were grown with complex medium or AMM and inoculated with fresh spores with a concentration of $5x10^6$ spores/mL. Static cultures were grown in complex media at 30 °C for three days. Planktonic cultivations of *A. nidulans* strains were grown in AMM at 37 °C for 24 hours at 200 rpm. For xylose inducing conditions, media were supplemented with 2 % (w/v) xylose. Data shown in figure 3 were obtained with *A. nidulans* cultures grown in static complex medium supplemented with 2 % (w/v) xylose.

Fungal molecular biology techniques

For polymerase chain reactions (PCR), 2x high fidelity Phusion master mix (Life Technologies, Darmstadt, Germany) was used unless otherwise noted. *A. nidulans* transformations were carried out according to standard protocols. *A. nidulans* RMS011 protoplasts were transformed as previously reported.[26, 27]Strains generated in this study are shown in Table S1. Transformants were verified for correct deletions by PCR (data not shown) and Southern blot analysis (Figures S1, and Tables S3 and S4).

Genome mining

The gene coding for the PKS *ausA* from *A. calidoustus* was used as probe. Blast analysis[28] was performed against the *A. calidoustus* and *P. brasilianum* open reading frame (ORF).[15, 16] AusA orthologues were identified in both species (Table S1). Putative ORFs surrounding *ausA* in *A. calidoustus* were retrieved and subsequently blasted against the *A. nidulans* and *P. brasilianum* ORF databases. The nucleotide identity among the different clusters was investigated using AlignX from Vector NTI (Invitrogen) (Table S1).

Extraction and HPLC–HRMS analysis

Austinoids were extracted from *A. calidoustus*, *A. nidulans* and *P. brasilianum* cultures as previously described.[10] High–resolution mass spectrometry (HRMS) was conducted on a Thermo Fischer Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometer with an electrospray ion source and an Accela HPLC system (Thermo Fisher Scientific) equipped with a C18 column (Accucore 2.6 µm 150 x 2.1 mm). Mobile phase 0.2 ml min^{-1}: water (0.1 % (v/v) formic acid): acetonitrile (0.1 % (v/v) formic acid), start 95:5 then to 2:98 in 10 minutes, four minutes 2:98, 0.1 minutes 95:5, 6.9 95:5; 20 minutes total run.

Plasmid assembly

All plasmids used in this study are listed in table S5. Further information is available in the online supplementary text.

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ASSOCIATED CONTENT

Supporting information

The Supporting Information is available free of charge via the Internet.

Experimental details regarding the assembly of the plasmids along with supplementary figures and tables can be found in the supporting information. This includes Southern blots, nucleotide comparisons and tables detailing the strains used in this study, the primer list and plasmid list.

Competing interest

The authors declare competing financial interests: part of this work was filed for patent under EP15200500.5.

Figure legends

Figure 1. Proposed biosynthetic pathway for austinoids in the three austinoid producing strains (figure modified from Valiante et al.[10]). (A) The extended pathway begins with austinol **1** and dehydroaustinol **8,** which are the final products identified in the *A. nidulans* austinoid pathway indicated in sky blue. The pink background marks the products identified in *P. brasilianum*, which end with acetoxydehydroaustin **10**. AusP is marked in pink as it is only present in *P. brasilianum* and *A. calidoustus*, while the proteins marked in green are unique to *A. calidoustus*. The remaining genes are present and functional in the three analyzed species. (B) Extracted ion chromatogram (EIC) overlays of the different austinoids identified in the reported species. Blue chromatograms

refer to shunt products. Each peak corresponds to the following *m/z*: **1**, *m/z* 459.20 $[M + H]^+$; **2**, *m/z* 501.21 $[M + H]^+$; **3**, *m/z* 499.19 $[M + H]^+$; **4**, *m/z* 515.19 $[M + H]^+$; **5**, *m/z* 517.20 $[M + H]^+$; **6**, *m/z* 599.24 $[M + H]^+$; **7**, *m/z* 615.24 $[M + H]^+$; **8**, *m/z* 457.18 $[M + H]^+$; **9**, *m/z* 501.21 $[M + H]^+$; **10**, *m/z* 557.20 $[M + H]^+$; **11**, *m/z* 559.21 $[M + H]^+$; **12**, *m/z* 575.21 $[M + H]^+$; **13**, *m/z* 597.23 $[M + H]^+$.

Figure 2. Austinoid gene clusters considerably vary in different fungal species. Genes depicted in sky blue represent common genes amongst the three species; pink portrays genes present only in *A. calidoustus* and *P. brasilianum*; cluster genes identified in *A. nidulans* and *A. calidoustus* are highlighted in yellow; unique genes in *A. calidoustus* are depicted in green; in grey are reported genes that are not involved in austinoid biosynthesis, including external bordering coding sequences. As reported, all austinoid biosynthetic genes in *A. calidoustus* are located in a single cluster, while in *A. nidulans* and *P. brasilianum* they are found in two distinct clusters. The cluster in *A. nidulans* seems to have been split into two pieces. This event likely determined the loss of genes involved in the biosynthetic pathway, such as *ausP* and *ausT*. The identified cluster in *P. brasilianum* was not only split, but some genes were also inverted compared to the *A. calidoustus* and *A. nidulans* gene clusters (*e.g. ausJ-ausK* and *ausE*). A residual pseudogene similar to *ausV* was identified in both *A. nidulans* and *P. brasilianum*.

Figure 3. Rewiring of the austinoid pathway in *A. nidulans*. (A) Austinoid gene cluster comparison between *A. nidulans* and *A. calidoustus*. (B) Chromatograms of compounds extracted from *A. nidulans* transformed with the $argB^+$-encoding empty vector grown as a planktonic culture (left panel) and static culture (right panel). (C) *A. nidulans* transformed with *ausP* led to the formation of 7-hydroxydehydroaustin **4** and related shunt pathways in planktonic cultures, demonstrating that the *A. nidulans ausQ* and *ausR* genes were still expressed and functional. (D) Transformation of *A. nidulans* with *ausP* and *ausT* led to the formation of **5** in planktonic cultures. (E) Expression of the

nonreducing PKS *ausV* in combination with *ausP* promoted the formation of the shunt product 1,2-dehydro-precalidodehydroaustin **13** in static cultures. (F) Expression of *ausV* and *ausT via* a polycistronic mRNA in combination with *ausP* made the biosynthesis of the majority of the *A. calidoustus* austinoid shunt pathways in *A. nidulans* possible during static growth. (G) The end product calidodehydroaustin **7** identified in *A. calidoustus* was finally produced in *A. nidulans* by co-expression of *ausP* with a polycistronic mRNA encoding *ausO*, *ausT* and *ausV* in static cultures. Blue chromatograms refer to shunt products. The EICs were produced as in Figure 1.

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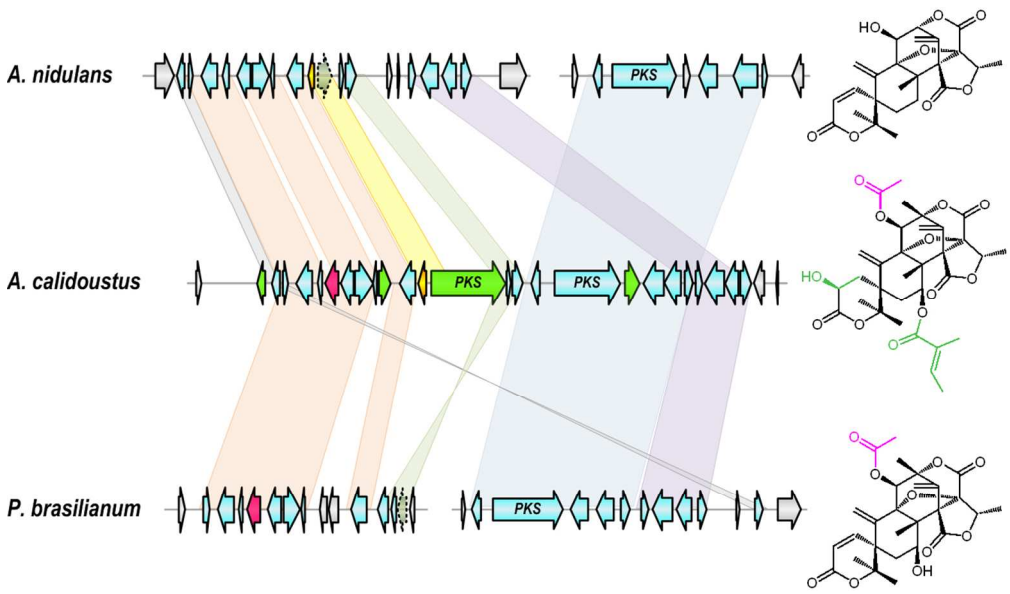

graphical abstract

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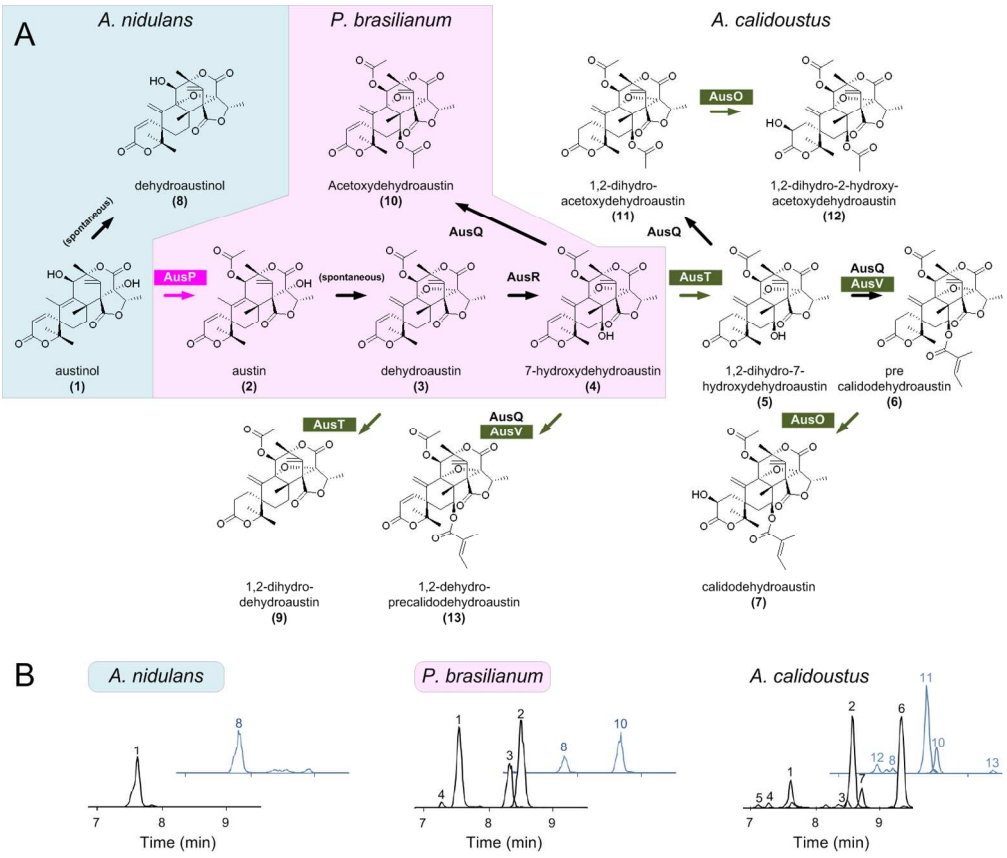

Proposed biosynthetic pathway for austinoids in the three austinoid producing strains (figure modified from Valiante et al.10). (A) The extended pathway begins with austinol 1 and dehydroaustinol 8, which are the final products identified in the A. nidulans austinoid pathway indicated in sky blue. The pink background marks the products identified in P. brasilianum, which end with acetoxydehydroaustin 10. AusP is marked in pink as it is only present in P. brasilianum and A. calidoustus, while the proteins marked in green are unique to A. calidoustus.The remaining genes are present and functional in the three analyzed species. (B) Extracted ion chromatogram (EIC) overlays of the different austinoids identified in the reported species. Blue chromatograms refer to shunt products. Each peak corresponds to the following m/z: 1, m/z 459.20 [M + H]+; 2, m/z 501.21 [M + H]+; 3, m/z 499.19 [M + H]+; 4, m/z 515.19 [M + H]+; 5, m/z 517.20 [M + H]+; 6, m/z 599.24 [M + H]+; 7, m/z 615.24 [M + H]+; 8, m/z 457.18 [M + H]+; 9, m/z 501.21 [M + H]+; 10, m/z 557.20 [M + H]+; 11, m/z 559.21 [M + H]+; 12, m/z 575.21 [M + H]+; 13, m/z 597.23 [M + H]+.

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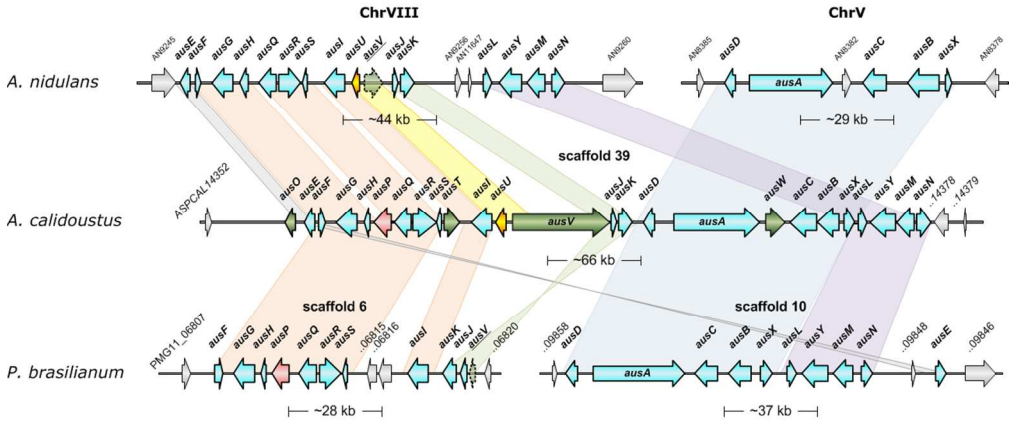

Austinoid gene clusters considerably vary in different fungal species. Genes depicted in sky blue represent common genes amongst the three species; pink portrays genes present only in A. calidoustus and P. brasilianum; cluster genes identified in A. nidulans and A. calidoustus are highlighted in yellow; unique genes in A. calidoustus are depicted in green; in grey are reported genes that are not involved in austinoid biosynthesis, including external bordering coding sequences. As reported, all austinoid biosynthetic genes in A. calidoustus are located in a single cluster, while in A. nidulans and P. brasilianum they are found in two distinct clusters. The cluster in A. nidulans seems to have been split into two pieces. This event likely determined the loss of genes involved in the biosynthetic pathway, such as ausP and ausT. The identified cluster in P. brasilianum was not only split, but some genes were also inverted compared to the A. calidoustus and A. nidulans gene clusters (e.g. ausJ-ausK and ausE). A residual pseudogene similar to ausV was identified in both A. nidulans and P. brasilianum.

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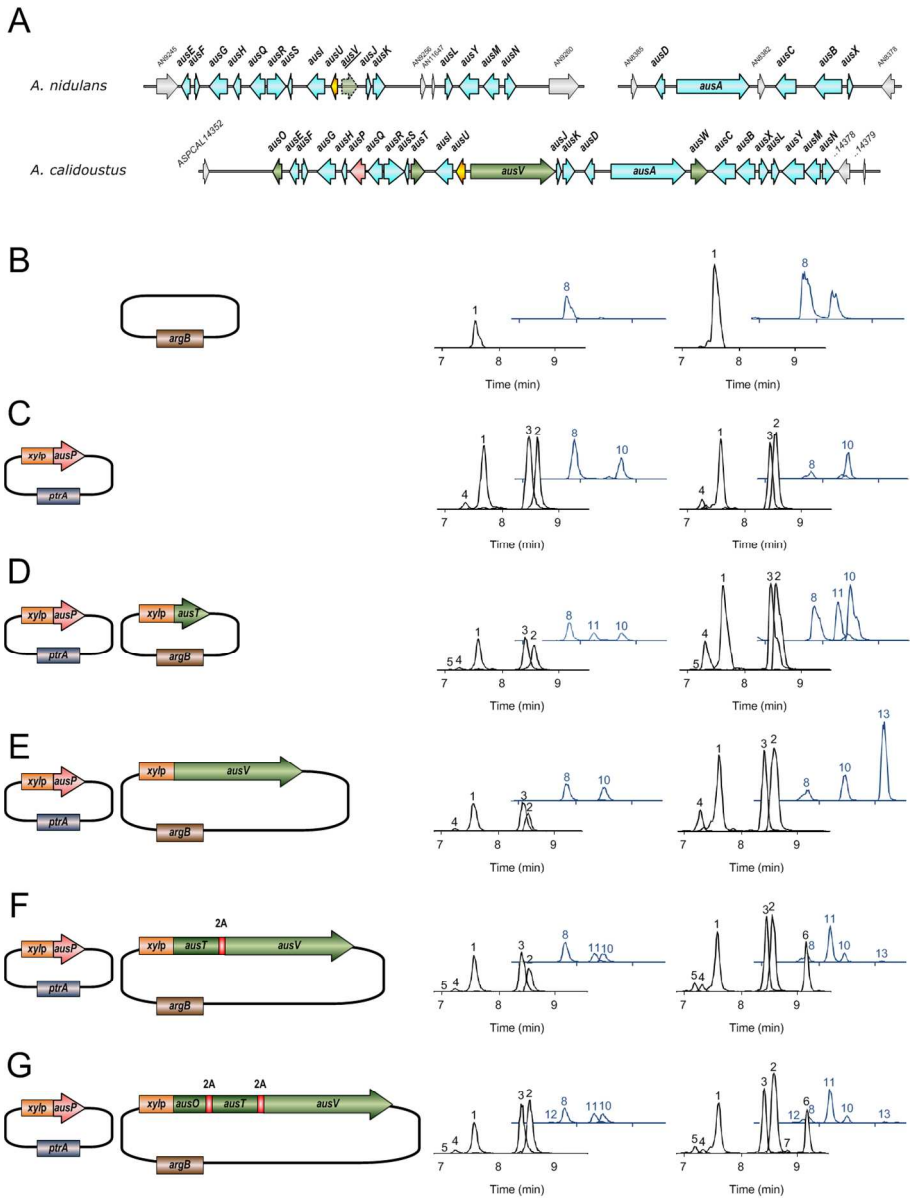

Rewiring of the austinoid pathway in A. nidulans. (A) Austinoid gene cluster comparison between A. nidulans and A. calidoustus. (B) Chromatograms of compounds extracted from A. nidulans transformed with the argB+-encoding empty vector grown as a planktonic culture (left panel) and static culture (right panel). (C) A. nidulans transformed with ausP led to the formation of 7-hydroxydehydroaustin 4 and related shunt pathways in planktonic cultures, demonstrating that the A. nidulans ausQ and ausR genes were still expressed and functional. (D) Transformation of A. nidulans with ausP and ausT led to the formation of 5 in planktonic cultures. (E) Expression of the nonreducing PKS ausV in combination with ausP promoted the formation of the shunt product 1,2-dehydro-precalidodehydroaustin 13 in static cultures. (F) Expression of ausV and ausT via a polycistronic mRNA in combination with ausP made the biosynthesis of the majority of the A. calidoustus austinoid shunt pathways in A. nidulans possible during static growth. (G) The end product calidodehydroaustin 7 identified in A. calidoustus was finally produced in A. nidulans by co-expression of ausP with a polycistronic mRNA encoding ausO, ausT and ausV in static cultures. Blue chromatograms refer to shunt products. The EICs were produced as in Figure 1.

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