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Black perithecial pigmentation in *Fusarium* species is due to the accumulation of 5-deoxybostrycoidin-based melanin

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Biosynthesis of the black perithecial pigment in the filamentous fungus *Fusarium graminearum* is dependent on the polyketide synthase PGL1 (\(oPKS3\)). A seven-membered PGL1 gene cluster was identified by over-expression of the cluster specific transcription factor \(pglR\). Targeted gene replacement showed that \(PGL1, pglJ, pglM\) and \(pglV\) were essential for the production of the perithecial pigment. Over-expression of \(PGL1\) resulted in the production of 6-O-demethyl-5-deoxybostrycoidin (1), 5-deoxybostrycoidin (2), and three novel compounds 5-deoxybostrycoidin anthrone (3), 6-O-demethyl-5-deoxybostrycoidin anthrone (4) and purpurfusarin (5). The novel dimeric bostrycoidin purpurfusarin (5) was found to inhibit the growth of *Candida albicans* with an \(IC_{50}\) of 8.0 ± 1.9 \(\mu\)M. The results show that *Fusarium* species with black perithecia have a previously undescribed form of 5-deoxybostrycoidin based melanin in their fruiting bodies.

The sexual development of the homothallic *Fusarium graminearum* (\(Fg\)) on wheat plants and in culture is well-described\(^1,2\). Perithecia (fructing body) formation can be induced *in vitro* by cultivating the fungus on special media, typically based on plant material, such as carrot\(^3\). The mature perithecia are flask-shaped, 140–200 \(\mu\)m in diameter, with an ostiole at the top\(^2\). The periderm of the perithecia consists of three layers distinguishable by light microscopy. The outer layer is two to three cells thick and consist of thick-walled, highly vacuolated spherical cells that accumulate a blue-violet pigment of unknown structure\(^2\) (Fig. 1). This pigment gives the perithecia their black appearance on the macroscopic scale, a feature that is shared by all members of the former *Gibberella* genus\(^4\). The function of the pigment is unknown, but protection of the ascospores inside the perithecium from UV radiation and reactive oxygen species or inhibition of ascospore germination has been suggested\(^5–8\).

Disruption of the 15 type 1 iterative polyketide synthase (PKS) encoding genes in *Fg* PH-1, has previously shown that \(oPKS3 (PGL1)\) was essential for production of the blue-violet perithecial pigment\(^5\). The \(PGL1\) gene is under a tight regulation in *Fg*, and expression is detected during late perithecium development coinciding with black pigmentation\(^7–11\). Deletion of the \(PGL1\) ortholog in *Fusarium verticillioides* (\(Fve\), former *Gibberella* member) also resulted in albino perithecia\(^5\). Proctor *et al.* suggest that the \(PGL1\) gene is part of a gene cluster, based on gene synten in the genomes of *Fg*, *Fve* and the more distantly related *Fusarium solani* (\(Fs\)). *Fs* and other members of the former *Nectria* genus are characterized by their red perithecia, a trait that has been linked to the activity of \(pksN\) and not \(PGL1\)\(^6\). The product of \(Fs\) PGL1 has been shown to be 3-acetonyl-1,6,8-trihydroxy-2-naphthaldehyde (6-O-demethylfusarubinaldehyde) by heterologous expression of the gene in *Aspergillus oryzae*\(^12\). This compound display a cyclization pattern identical to the pattern predicted for formation of fusarubins.

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is approximately twice the size of that found in (Fig. 2A). The insert in Fvi, Fs pglX Fg based on the available data, to determine whether the inserts found in the, Fs ison revealed that the sequence contained a partial duplication of the Fvi results to those reported by Brown and co-workers16.

evolutionary steps towards assembly or breakup of the cluster.

ble for perithecial pigmentation. Fusarubins are characterized by their yellow to red colors at physiological pH14, a chemical composition of the pigments in perithecia from Fusarium sp.

Results

The analyzed Fusaria sp. all contain the PGL cluster. Comparative genomics of eight genome sequenced Fusarium sp., using Shuffle-LAGAN alignment of the genomic regions surrounding the PGL1 loci, revealed extensive sequence synteny (Fig. 2A). The syntenic region included seven genes, found in all analyzed species, suggesting that the core PGL1 gene cluster consist of PGL1, pglJ, pglM, pglX, pglV, pglR and pglE (Fig. 2B). The relative orientation of the genes was conserved across the species. However, Fg and Fusarium pseudograminearum (Fp) contained a 2.3 kb insert located between the pglM and pglX genes (Fig. 2B), a region that includes Fg-pglL. PglL is predicted to encode adenylosuccinate lyase (Ade13), a key enzyme in the central metabolism. As no other orthologs were found in the Fg genome we hypothesize that pglL is not part of the cluster15. In Fs and Fusarium virguliforme (Fvi), both members of the former Nectria genus, larger inserts are also found between pglM and pglX (Fig. 2A). The insert in Fs is approximately twice the size of that found in Fvi, and a dot-plot based comparison revealed that the Fs sequence contained a partial duplication of the Fvi sequence. It is currently impossible, based on the available data, to determine whether the inserts found in the Fg, Fs, Fp, Fi and Fvi clusters represent evolutionary steps towards assembly or breakup of the cluster.

Analysis for functional domains in the encoded enzymes (Supplementary file Table S1) yielded very similar results to those reported by Brown and co-workers15.

pglR encodes a PGL1 cluster-specific transcription factor. Analysis of the available Affymetrix expression data18 for perithecia development in Fg showed that six conserved genes: pglM, pglJ, pglX, pglV, pglR, and pglE were co-regulated with PGL1 and that expression of this putative cluster peaked at 96 hours after induction of perithecum formation (Fig. 3A). The cluster includes a putative transcription factor encoding gene pglR (FG09188) (Table S1). The role of PglR in regulation of the cluster was examined by introducing an additional copy of the pglR under the control of the constitutive Aspergillus nidulans GAPDH promoter, into the PKS12 locus. Agrobacterium-mediated transformation (AMT) of the expression cassette resulted in 45 EO-pglR transformants, of which two were verified by diagnostic PCR and Southern analysis (Supplementary File 1). Overexpression of pglR did not affect perithecum formation or color, but the vegetative mycelium displayed a yellow-brown pigmentation not observed in the white reference strain (FgHUEA:ΔPKS12) (Fig. 4A,B). Cultivation of the strain in liquid DFM media showed that the novel pigments were excreted and soluble in the aqueous solution.

Expression analysis of the putative PGL1 gene cluster, by semi-quantitative RT-PCR, showed that PGL1, pglM, pglJ, pglX, pglV and pglR were up-regulated in vegetative mycelium of the EO-pglR strain compared to the wild type, while expression of pglE was not affected (Fig. 3B). These results show that PglR is the positive acting pathway specific transcription factor for the PGL1 cluster consisting of the six genes.

Identification of a putative binding motif for PglR. A search for potential palindromic transcription factor binding sites in the five promoter regions of PglR regulated genes resulted in the identification of a CGGN,CCG motif, which was significantly overrepresented (P = 2.5e-07), e.g. the motif occurred nine times (1.9 time/kb) in the promoters in the Fg cluster, while the background occurrence of the motif was 0.10 times/kb in all Fg promoters (Fig. 2B). Similar results were obtained for Fusarium oxysporum (Fo) and Fve. The motif was found in four of the five promoter regions, and the locations of these potential binding motifs were largely conserved.

Figure 1. Fg perithecia. (A) Fg wild type on carrot agar. (B) Crushed perithecium showing the purple pigmentation of the periderm - the vegetative mycelium (red) is visible to the right. (C) Purified purpurfusarin in DMSO.
across the different species (Fig. 2B), except in Fs and Fvi where none was found in the promoters of plgM and plgV. The conservation in placement of the motif and the significant overrepresentation suggests that the sites are under active selection, supporting a biological function.

**The O-PGL1 and EO-pglR strains produced novel compounds.** Efforts to identify and characterize pigments directly from perithecia failed as minute amounts were obtained. As an alternative we used genetic engineering to overexpress the involved genes in the vegetative mycelium of the fungus. To determine the primary product of Fg-PGL1 we exchanged the endogenic perithecial specific Fg-PGL1 promoter with the constitutive A. nidulans GAPDH promoter. A total of 42 transformants were isolated of which two were verified by diagnostic PCR and Southern analysis (Supplementary File 1). Introduction of the constitutive promoter in front of Fg-PGL1 resulted in expression of the gene in the vegetative mycelium, and had no detectable effects on the expression of the neighboring genes belonging to the cluster (Fig. 3C). The mycelium of the O-PGL1 strain displayed a brown phenotype, which differed significantly from the red color of the wild type (Fig. 4A). The strain produced perithecia at a similar rate and appearance as the wild type (Fig. 4A). Cultivation of the O-PGL1 strains in liquid media (DFM and YPG) resulted in a brown coloring of the culture broth, which was not observed for the wild type (Fig. 4B).

UHPLC-DAD-HRMS based analysis of the filtered culture broth, from 7-day old O-PGL1 and EO-pglR cultures, revealed five compounds not present in the wild-type (Fig. 5A). Compounds (2) and (3) were found in

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**Figure 2.** Identification of the core PGL1 gene cluster and possible transcription factor binding sites. (A) Shuffle-LAGAN alignment of the putative PGL1 gene cluster from eight different fusaria species with Fvi as a reference sequence (top). The graphs show the level of % identity between the given species and Fvi, shown from 50% to 100% calculated using a 100 bp sliding window. (B) The core PGL1 gene clusters consisting of PGL1, pglI, pglM, pglX, pglV, pglR and pglE. Fg and Fp contains an additional gene pglL, larger inserts are found at the same site in the Fs and Fvi clusters (Fs contain a duplication of the Fvi sequence). Analysis of the promoter regions identified a single palindromic sequence (CGGNCCG) to be significantly enriched. The location of this potential transcription factor binding motif is largely conserved across the different species, except for Fs and Fvi where none is found in the plgM and plgV promoters.
the EO-pglR strains, while the O-PGL1 strain produced all five compounds (1)–(5). The elemental composition and UV spectra of the five compounds all suggested highly conjugated compounds, consistent with that of polyketide derived pigments. Extracted ion chromatograms for the five identified compounds showed that the wild-type did not produce any of the compounds in the vegetative mycelium. This supports that production of the five novel compounds in the overexpression strains were the result of switching on one or more of the PGL1 genes. Dereplication resulted in tentative identification of compound (1) and (2) as 6-O-demethyl-5-deoxybostrycoidin (1) and 5-deoxybostrycoidin (2), respectively. Their structures were later confirmed by NMR spectroscopy, comparing the obtained data with those found in the literature17. In addition to compound (1)–(5) the concentration of several other compounds was found to increase in the mutants compared to the wild type strain (Fig. 5A). Extracted ion chromatograms of these compounds showed that they were all produced at low concentrations in the vegetative mycelium of the wild type. This indicates that their production is independent of the PGL1 gene which is silent in the vegetative mycelium of the wild type (Fig. 3C).

Structural elucidation of 5-deoxybostrycoidin anthrone (3). The effort to identify compound (3) by dereplication was unsuccessful. Its [M+H]+ was found to be 256.0965 m/z, and its elemental composition was calculated to C15H13NO3 (theoretical [M+H]+ 256.0968). Examination of the 1D NMR showed that all signals had a minor peak in a ratio of 3:1.8. The reported structure is that of the major peaks. The 1D 1H-NMR consisted of one aromatic methyl group at 2.64 ppm (H-15), one methyl ether group at 3.87 ppm (H-16), four aromatic protons at 6.24 (H-7), 6.44 (H-5), 7.17 (H-4) and 9.35 (H-1), one aromatic hydroxyl at 13.17 ppm (OH-8) and lastly two protons at 4.24 ppm (Table S4). In addition, the two protons H-5 and H-7 appeared to be meta-coupled (J = 2 Hz). The main difference in the 1D NMR spectra between the proposed structure of (3) and the structure of (2) was found in the signal of H-10, this signal at 4.24 ppm and C-10 at 31.5 ppm was found to be in good agreement with what has been reported for emodin anthrone18. The minor peaks were consistent with the tautomer of (3) the 5-deoxybostrycoidin anthrol (Fig. 5C).

Structural elucidation of 6-O-demethyl-5-deoxybostrycoidin anthrone (4). From the O-PGL1 strain an unknown compound with a [M+H]+ of 242.0711 m/z was isolated. The chemical formula was calculated to C14H11NO3 (theoretical [M+H]+ 242.0812). The UV spectrum was identical to that of (3). In addition, the
Figure 4. Phenotypes of the generated *Fg* strains. (A) Cultivated on solid DFM medium and perithecium formation on carrot agar. (B) Medium fraction from liquid cultures of wild type, Δ*PGL1*, *EO-*pglR and O-*PGL1* strains cultivated for ten days in liquid DFM. The medium was filtered and centrifuged to remove cell debris and non-soluble metabolites.
chemical formula only differed by a single CH3 suggesting (4) to be the 6-O-demethylated form of (3) (Fig. 5C). Due to very low amounts of the compound, only 1D NMR was performed. The examination of the 1D NMR of (4) in DMSO-d6 showed one aromatic methyl group at 2.32 ppm (H-15), five aromatic signals at 5.93 (H-5 or H-7), 6.25 (H-7 or H-5), 6.31 (H-10 or H-5), 6.90 (H-5 or H-10), 8.64 (H-1) and two aromatic hydroxyl groups.
Structural Elucidation of Purpurfusarin (5). The purple compound (5) was only observed in the O-PGL1 strain. The monoisotopic m/z of its [M + H]+ ion was 477.1080 Da, corresponding to an elemental composition of C28H16N2O6 (theoretical [M + H]+ 477.1081 m/z). Examination of the 1H spectrum revealed 6 singlet resonances: one aromatic methyl group at 2.33 ppm (H-15/15′), three aromatic resonances at 6.39 (H-7/7′), 7.72 (H-4/4′) and 9.43 (H-1/1′) ppm and two phenolic resonances at 15.86 (OH-8′/8′′) and 18.47 (OH-6/6′′) ppm (Table S5). An interesting observation was that the extreme downfield hydroxyl proton had an odd signal intensity ratio to the aromatic protons and methyl group being 1:2:6. This suggested that this hydroxyl group was partly deprotonated or exchanged. Examination of the 13C spectrum revealed 14 resonances, all of which could be accounted for in the HMBC and HSQC. The mass spectrometric analysis gave an elemental composition of the molecule with 28 carbon atoms, (5) indicating a highly symmetrical compound. The aromatic proton signals at 7.72 and 9.43 indicated the proximity of an aromatic nitrogen, which was confirmed by 15N-HMBC: both H-1/H-1′ and H-4/H-4′ as well as the methyl protons at 2.73 ppm correlated to a nitrogen at 317.5 ppm (relative to external liquid ammonia at 25 °C).

The elucidation of (5) was aided by the observed similarity between 1H and 13C shifts of (1) and (5) (Table S5) and those reported for bostrycoidin19. Especially, the chemical shift values in the nitrogen containing ring were directly comparable. As the NMR and HRMS suggested a dimer, we hypothesize that (5) is a fusion product of the molecules (1) and (4). The fusion of two anthrones via oxidative coupling has similarly been described as a putative mechanism in the biosynthesis of prototricypenic and hypericin found in St. John's wort20. The oxidative coupling of two anthrones could give rise to two different isomeric configurations, C1 and C2 as shown in Supplementary File 1. However, the SELNOE (1D selective NOESY) spectra made by individual irradiation of all 1H-resonances except for the two downfield hydroxyl protons, and the 2D NOESY spectra, showed (merely) a strong correlation between the methyl group H-15/H-15′ and H-4/H-4′ and a comparatively weak correlation between H-15/H-15′ and H-1/H-1′, while no correlation between H-4/H-4′ and H-7/H-7′ or H-4/H-4′ and 6/6′-OH could be observed, which would have been expected if 5 had adopted the isomeric configuration C2 Hence, we expect (5) to adopt the C1 configuration. This is further supported by Falk et al.20 reporting that titrating hypericin with KOH generated a hypericinate ion with a characteristic hydroxyl resonance at 18.37 ppm exhibiting a 1:2:6 signal intensity ratio, similar to what we observed in (5). Hence, we suggest that (5) adopt the C1 configuration as seen in prototricypenic (Fig. 5C).

PGL1, pglJ, pglM and pglV are involved in perithecial pigment production. Targeted replacement of pglJ, pglM, pglV, pglX and PGL1 by AMT resulted in 40 ΔPGL1, 42 O-PGL1, 15 ΔpglJ, 21 ΔpglM, 30 ΔpglV and 39 ΔpglX hygromycin resistant transformants. The PCR and Southern based analysis of the isolated transformants verified the desired modification and single copy integration of the T-DNA (Supplementary File 1). For each mutant type, a single verified transformant was selected for further phenotypical characterization. The ΔPGL1 strain produced white perithecia on carrot agar plates (Fig. 4A), as previously reported by Gaafoor et al.7. Targeted replacement (ΔpglJ, ΔpglM, ΔpglV) resulted in perithecia with an altered pigmentation compared to the wild type (Fig. 4A). The ΔpglJ and ΔpglM strains had light yellow perithecia, and the ΔpglV strain dark brown perithecia. Replacement of pglX did not visibly affect peritheccium pigmentation. Chemical analysis, by targeted UHPLC-DAD-HRMS of isolated perithecia from the generated ΔPGL1 deletion strain, supported the visual observations (Fig. 5B). Compounds (1) and (2) were detectable (EIC view) in extracts from the wild type perithecia while neither was found in the ΔPGL1 strains. A similar search for fusarubin based metabolites did not reveal any peaks, showing that the blue-violet pigments in the Gibberella type of perithecia must be based on bostrycoidins and not fusarubin metabolites.

Antifungal susceptibility testing. The IC50 of (5) and the positive reference yanuthone D for C. albicans was extrapolated from compound specific dilution sequences and annotated as the average concentration for which 50% inhibition plus minus the standard deviation was observed. Purpurfusarin was found to have an IC50 of 8.0 ±/− 1.9 μM and yanuthone D with an IC50 of 3.3 ±/− 0.5 μM.

Discussion
The PGL1 gene strain was found to consist of six core genes that were highly conserved in the analyzed Fusarium species (Fig. 2B). Overexpression of PglR confirmed that the cluster was regulated by PglR, as also described for Frp11. Analysis of promoter regions in PglR co-regulated genes revealed a significant enrichment of the CGG-N1-CGG motif. The Zn(II)Cys6 family of transcription factors often bind to short palindromic sequences consisting of inverted trinucleotide repeats separated by a variable length spacer.21. The identified CGG repeat is also seen in a number of other Zn(II)Cys6 transcription factors, such as GAL4 (CGG-N1-CGG)22, War1 (CGG-N2-CGG)23 and Rds1 (CGGCGCG)24, which suggest a common mode of protein-DNA interaction and that PglR binds as a homodimer. The overrepresentation and the conserved position across the species make the motif a strong candidate for a PglR binding motif.

Targeted deletion of PGL1, pglJ, pglM or pglV in Fg impacted the peritheccium color (Fig. 4A), showing that the encoded enzymes are required for biosynthesis of the pigment. Transcriptional activation of PGL1 resulted in the formation of five pigments (1)–(5) in the mycelium, while activation of the entire cluster by pglR overexpression led to the accumulation of (2) and (3). The identified compounds all belong to the bostrycoidin family that has not previously been reported in Fg and three of the formed compounds (3–5) are completely novel. Based on the
structure of the identified compounds and the biosynthetic potential of the involved enzymes, we formulated a model for the biosynthetic pathway (Fig. 6). This includes two alternative routes for the formation of (2), the most decorated of the compounds. Based on the results of Awakawa et al. the primary product of PGL1 was expected to be 6-O-demethyl-fusarubinaldehyde. However, the simplest compound detected in the O-PGL1 strain was the nitrogen-containing compound (4). Standard polyketide biosynthesis does not offer an explanation for the introduction of nitrogen as observed in bostrycoidins. Parisot et al. have, however, previously shown that bostrycoidins can be formed at room temperature from ‘anhydrofusarubin lactol’ when reacting with ammonia (50% after 72 hours at room temperature), making it likely that the compound is also formed spontaneously in vivo. Alternatively, the nitrogen atom is introduced by aminotransferase activity that transfers the amine group into the terminal aldehyde of 6-O-demethyl-fusarubinaldehyde similarly to what has been proposed by Wagoner et al.

Formation of (1), (2), and (3) in the O-PGL1 strains shows that the vegetative mycelium contains enzymes that are capable of converting the primary PKS product (4) to (1)–(3). Expression of the entire PGL1 gene cluster eliminated accumulation of the early pathway intermediates (1) and (4), showing that the cluster encoded enzymes can outcompete the shunt reaction in the mycelium. We propose that the linking of compounds (1) and (4) to yield

Figure 6. Proposed biosynthetic pathway for the formation of 5-deoxybostrycoidin. Compounds in brackets are predicted intermediates. Compound 3 and 5 are likely shunt products formed upon expression in the mycelium tissue.
the dimeric compound (5) is likely to proceed via an aldol type of condensation, followed by generation of the core double bond by loss of water and finally phenolic oxidative coupling (Fig. 6), possibly catalyzed by the GIP1 laccase from the aurofusarin gene cluster, which is known to be active in the mycelium and modifies compounds with similar structural features.

Though (5) was the only compound with a blue-violet color, similar to that found in perithecia, it was not itself detectable in perithecia (Fig. 5), suggesting that it is a shunt product only formed as a result of the modified expression pattern. However, the detection of (1) and (2) in wild-type perithecia suggests that these form the basis for formation of the unextractable blue-violet pigment, which could be either a polymer or compounds covalently linked to the cell wall. A situation that resembles what has been reported for other fungal pigments, such as DHN-melanin.

Many of the intermediates from the fusarubin/bostrycoidin pathways have previously been shown to display a wide range of biological activities, which include antibiotic, fungicidal, insecticidal and herbicidal activities, reviewed by Pariset et al. As part of a larger screen, we tested the bioactivity of (5) against C. albicans and found that it had an IC50 of 8.0+/−1.9 μM; in comparison the positive standard (yanuthone B) in the experiment had an IC50 of 3.3+/−0.5 μM, while the less potent of the tested compounds had an IC50 >100 μM. The available amounts of the two other novel compounds, (3) and (4), did not allow for a similar test.

**Fusarium** sp. are characterized by producing either of three different red mycelium pigments; aurofusarin (oPKS12/AUR), bikaverin (oPKS16/BIK1) or fusarubin (oPKS3/PGL1). The identified role of the PGL1 gene cluster in Fg perithecia pigmentation and its formation of 5-deoxybostrycoidin likely extends to other members of the former Gibberella genus. The role of the cluster in mycelium fusarubin formation, in members of the former **Nectria** genus, indicates that the cluster has undergone a dramatic shift in function during evolution. PGL1 orthologs are found in all genome sequenced *Fusaria* sp., while the PKS (pksN) responsible for the red perithecial pigment is only found in members of the former **Nectria** genus (Fv: contig AEYB01000515 and AEYB01000516).

This high level of diversity, with respect to the combinations of pigments and their production patterns, is surprising. It could be argued that difference in pigment use is due to adaptation to different ecological niches, but species with different pigment profiles have been shown to inhabit the same niche. A more plausible hypothesis is that the various polyketide pigments (fusarubin, bostrycoidin, bikaverin, aurofusarin and the uncharacterized red pigment from **Nectria** perithecia) are functionally redundant as they are all naphthoquinones capable of redox cycling and have overlapping absorption spectra. Functional redundancy based on two or more genes efficiently eliminates active selection on the genes. Nowak and coworkers have identified several evolutionary scenarios that allow for persistence of redundant genes, but in the majority of cases this situation results in random elimination of either of the redundant genes (systems) or a split of the shared function between the involved genes, to yield a genetically stable situation. The split can be accommodated by adapting different expression patterns, with respect to time or tissue, or by the evolution of novel non-overlapping functions. The occurrence of the PGL1 cluster in all sequenced *Fusaria* sp. suggests that this is the ancestral pigment system, compared to the oPKS12, oPKS16, and pksN systems that are only found in subclades of the *Fusarium* genus. The PGL1 gene cluster may originally have been responsible for both mycelium and perithecial pigmentation, but the acquisition of a redundant pigment system led to a change in the PGL1 clusters expression to accommodate two parallel pigment biosynthetic pathways for mycelial and perithecial pigmentation. Subsequent introductions of new pigment systems would result in replacement of old functionalities, see evolutionary models in Supplementary File 1. The alternative pigment systems were likely acquired by horizontal gene transfer events, involving entire gene clusters. The ‘division of labor’ model is supported by the observation that deletion of oPKS12, oPKS16, PGL1 and pksN all result in albino tissues, and that reported expression of the Ff-PGL1 cluster in the Ff mycelium occurs under conditions where the normal mycelial pigment bikaverin is not produced. The ‘division of labor’ model does not depend on absolute division to be genetically stable, but simply that the individual components each have one unique non-overlapping function, which would allow for situations where multiple pigment systems are active in the same tissues.

The conservation, replacement and development of redundant pigment systems strongly indicates that pigmentation plays a key to the survival of members of the *Fusarium* genus.

**Conclusion**

The present study for the first time provides direct evidence that the black perithecial pigmentation in *Fusarium graminearum* is due to the accumulation of a 5-deoxybostrycoidin based melanin, and not as previously proposed fusarubins. A situation that likely extends to other *Fusarium* sp. with black perithecia, e.g. members of the former Gibberella genus. Synthesis of the detected 5-deoxybostrycoidin is based on a six-membered gene cluster, expression is controlled by the pathway specific transcription factor PglR. The study also offers an insight into the evolutionary forces that has shaped secondary metabolism of filamentous fungi in general. The existence of highly diverse pigment systems within the *Fusarium* genus can likely be explained by multiple horizontal gene transfers, involving entire biosynthetic gene clusters, resulting in genetic instability due to functional redundancy between the clusters. A situation that has either been resolved by random elimination of one of the clusters, or by evolution in the clusters expression patterns to yield a genetically stable state. This model offers an explanation for how secondary metabolite gene clusters, in general, can be acquired and lost in an evolutionary perspective.

**Experimental Procedures**

**Microorganisms, Culture conditions and Genetic modifications.** *F. graminearum* PH-1 (NRRL 31084) wild-type was used as starting material for genetic modifications. *Agrobacterium tumefaciens* LBA4404 was used for Agrobacterium Mediated Transformation (AMT). Vectors for targeted replacement (pAg1-H3::ΔPGL1) and overexpression (pAg1-H3E::O-PGL1) of PGL1 were constructed by In-Fusion cloning, using the primers described in Table S2. Vectors for targeted replacement...
of pglL, pglM, pglX and pglIV were constructed via USER cloning as described in Frandsen et al.36. The inserts were verified by sequencing. The vector for overexpression of Fg-pglR was constructed by PCR amplifying the genes coding sequence and terminator using the primers pglR-E1/E2 (Figure S2), followed by USER cloning into pBF-HUEA37. AMT of Fg was carried out as described in Malz et al.32 with the modification described in Frandsen et al.32. Correct Fg transformants were identified by PCR-based screening, using four primer pairs (Table S2) and Southern analysis. Genomic DNA, for Southern analysis, was obtained following the procedure described in Malz et al.32.

Perithecia were produced on carrot agar plates. The cultures were incubated for seven days, at 20 °C with a continuous exposure to a mix of cool white light and fluorescent black light (Blacklight-blue F18W/BLB-T8 from SYLVANIA). Self-fertilization was induced by spreading 2 ml 2.5% aqueous Tween 60 per plate with a sterile Drigalsky spatula. The incubation continued for additional 10–14 days, to allow for perithecia formation.

**Enzymes, oligonucleotides, kits and apparatus.** The PfuTurbo Cx Hotstart DNApol (Stratagene) was used for USER cloning and Taq DNApol (Sigma) for screening and RT-PCR reactions. Restriction enzymes and USER enzymes were from New England Biolabs. Primers were from Invitrogen and MWG. Vector DNA was prepared from liquid cultures using the Qiagen Miniprep Kit. PCR products were purified using the GE Healthcare GFX clean-up system. Sequencing was performed by GATC Biotech AG (Constance, Germany).

**Genome sequences and comparative analysis of the PGL1 cluster.** Genome sequences for Fg, Fve and Fo were retrieved from FGDB at MIPS and http://www.broadinstitute.org/ff from GenBank (HE613440) with annotations from MIPS38. Fs from Nb 2.039, Fp CS3096 (AFNW00000000.1)40, Fvi (AYE01000000)41 and Fo 05001 (Fat05001) from Genbank42. The sequences were handled using CLC Main Workbench 7.0 (Qiagen).

PGL1 orthologs in the eight *Fusarium* genomes were identified by blastn analysis. The DNA sequences (60–100kb) surrounding the PGL1 locus were retrieved and aligned using the Shuffle-LAGAN global chaining algorithm43, with a sliding window of 100bp and the conservation level set to >70%44. The results were visualized with the mVISTA browser (http://genome.lbl.gov/vista/). Prediction of conserved functional motifs were performed using the Conserved Domain Database (CDD), Pfam and BRENDA45,46.

**Identification of putative binding sites for pglR (PGLR) in the PGL1 gene cluster.** The promoter regions of the genes in the putative cluster were analyzed for potential transcription factor binding sites using “Dyad-analysis” from “Regulatory Sequence Analysis Tool” (RSAT) (http://www.rsat.eu/)47. The background occurrence of motifs in promoters (1000bp upstream sequence) of all the predicted genes in Fg (13,332 genes), Fve (14,179 genes) and Fo (17,735 genes) were analyzed using the “DNA pattern” program at RSAT.

**Expression analysis of genes surrounding the PGL1 locus.** Affymetrix GeneChips for gene expression in Fg during peritheciun development in culture (accession no. FGS) was retrieved from PlexDB30. The dataset included data for six time-points during peritheciun development. Data for the 20 genes surrounding the PGL1 locus were retrieved as RMA-normalized data, and divided by the value at 0 H to show the change in expression relative to vegetative growth.

Vegetative mycelium for gene expression analysis was produced by cultivating triplicates of the wild type, O-PGL1 and EO-pglR strains for 5 days, in 50 ml liquid Yeast Peptone Dextrose (YPD medium) at 25 °C in 300 ml Erlenmeyer flasks at 150 rpm. The cultures were filtered through a Miracloth, and the mycelium was washed twice with sterile water, then frozen in liquid nitrogen. cDNA was synthesized as described in Malz et al.32. Primers, amplifying between 359 bp and 545 bp, were designed for locus FG09177.3 to FG09194.3 (Table S3). Genomic DNA from the wild type was used as a positive PCR control. PCR conditions were: 95 °C for 5 min, 25 × (95 °C for 30 sec, 60°C for 30 sec, 72 °C for 1 min) and a 72°C for 10 min. Expression of Fg-GAPDH was used as a reference gene to monitor the general gene expression level and to normalize the cDNA levels.

**UPLC-HRMS analysis of extracts.** Analytical LC-MS was performed using a Dionex Ultimate 3000 ultra-high performance liquid chromatography (UHPLC, ThermoFisher, Waltham, MA) equipped with a diode-array detector (DAD) system hyphenated to a maxiXs G3 Oa-TOF mass spectrometer (Bruker Daltonics, Billerica, MA). Samples were introduced with an injection volume of 1 μl for mass spectrometric analysis and 5 μl for recording of UV/VIS data. The separation was performed on a reverse-phase Kinetex C18 column (100 × 2.1 mm, 2.6 μm, Phenomenex, Torrance, CA, US). The column temperature was maintained at 40 °C. The mobile phase consisted of MilliQ treated H2O (A) and ACN (B) both containing 20 mM formic acid (FA). The analytes were eluted using a linear gradient, at a constant flow of 400 μl min⁻¹, having a starting composition of 10% B and increased to 100% B over 10 min. This composition was held for 3 min before returned to 10% B over 0.1 min, and held at this for 2.4 min to re-equilibrate the column.

The detection of the analytes was performed using an online DAD (Dionex Ultimate 3000), configured to detect from 200 to 600 nm, combined with an online maxiXs 3G Qq-Oa-TOF (Bruker Daltonics GmbH). The analytes were ionized using positive electrospray. The nebulizer gas was set to 2.4 bars; the drying gas flow was 12 ml/min and the drying temperature was 220°C. The capillary voltage was 3.4 kV. The MS was set to scan in full scan mode with a mass range of 100–1000 m/z. The MS was calibrated using sodium formate (Fluka analytical grade) applying the Bruker HPC (High Precision Calibration) algorithm infused prior to each sample run. Dereplication was performed using AntiBase 2010 (Hartmut Laatsch, Wiley-VCH) and an in-house database containing 972 natural compounds.

**Purification of novel pigments produced by overexpression strains.** Extraction and purification of Purpurafusarin (S): The O-PGL1 strain was grown in liquid YPD media for ten days. The cultivation broth was partitioned between EtOAc four times, and the combined organic layer was concentrated in vacuo. The crude
extract (620 mg) was fractionated on a normal phase 10 g diol column (ISOLUTE, BIOTAGE, Uppsala, Sweden). The column was eluted using different solvent systems, 15 ml at a time, from heptane to MeOH (Tables S6–8): A purple band trailed and co-eluted together with other dark compounds. This fraction (67.2 mg) was then re-run on a diol column (10 g), this time using a binary EtOAc/MeOH gradient. The purple compound eluted in two fractions that were pooled (8.4 mg). The final purification of the purple compound was achieved on a Sadasph LH-20 column (40 × 4 cm), equilibrated in MeOH. The column was eluted with MeOH with a linear flow rate of 3.2 cm/h. The separation was visually guided by the purple band. The pooled LH-20 fractions yielded 3.0 mg.

Extraction and purification of 5-deoxybostrycoidin anthrone (3): The EO-pglR strain was cultivated in liquid YPG media for ten days. The filtered broth was partitioned between EtOAc four times, and the combined phases were concentrated in vacuo given a dark yellow powder. The final purification of (3) was achieved on a LUNA PFP column (250 × 10 mm, 5 μm, Phenomenex, Torrance, CA, US). The column was eluted using a linear gradient consisting of MeOH and MilliQ H2O, both containing 20 mM FA. The gradient was 60–100% MeOH over 20 min. Two fractions were collected containing a yellow and a red compound. After evaporation of the solvent under a stream of nitrogen, the yellow fraction turned red. LC-HRMS of the two fractions showed that the compound eluted at the same times and the fractions were thus pooled, giving a total yield of 3.8 mg.

Extraction and purification of 6-O-Demethyl-5-deoxybostrycoidin anthrone (4): The O-PGL1 strain was cultivated in YPG liquid media for three days. The filtered broth was partitioned between EtOAc four times and the combined phases were concentrated in vacuo. The crude extract (144 mg) was fractionated on a 10 g Isolute Diol (see Table S for details). Compound (4) eluted in two fractions that were pooled to yield 33.8 mg. The final purification of (4) was achieved on a LUNA PFP column (250 × 10 mm, 5 μm, Phenomenex, Torrance, CA, US) in a 60–100% MeOH gradient containing 20 mM FA over 20 min. The yield was below 1 mg.

**Nuclear magnetic resonance (NMR) measurements.** All 1D and 2D NMR experiments were acquired on a Varian Unity Inova 500 MHz (Varian Inc., Palo Alto, California) or Bruker Avance AVII 600 MHz NMR spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) equipped with a cryoprobe and using standard pulse sequences. All samples were dissolved in deuterated DMSO-d6 or CDCl3.

**Antifungal susceptibility test.** The antifungal activity of purpurfasarin was tested towards Candida albicans in accordance with the CLSI standards in RPMI-1640 medium adjusted to pH 7 with 0.165 M MOPS buffer46. The inoculated media (2.5 × 10⁵ cells per ml) was transferred to 96 well microtiter plates in aliquots of 200 μl using a Hamilton STAR liquid handling workstation with an integrated Thermo Cytomat shaking incubator and Biotek Synergy Mx microplate reader. The test compounds were dissolved in DMSO and applied in concentrations ranging from 100 μM to 1.25 μM (Holm et al.29). The plates were incubated at 35 °C in an integrated shaking incubator under constant shaking at 1200 rpm with an amplitude of 2 mm. Optical density was automatically recorded every hour for 20 hours using the integrated plate-reader.

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Supplementary information

Additional Information

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Author Contributions

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R.J.N.F. and H.G. conceived the experimental design and wrote the manuscript. R.J.N.F. performed the molecular biological work. R.J.N.F. and E.L. performed the bioinformatics analysis. S.A.R., R.J.N.F., S.U., D.P., C.H.G. and R.J.N.F. and H.G. conceived the experimental design and wrote the manuscript. R.J.N.F. performed the molecular work. R.J.N.F. and E.L. performed the bioinformatics analysis. S.A.R., R.J.N.F., S.U., D.P., C.H.G. and R.J.N.F. and H.G. conceived the experimental design and wrote the manuscript.
