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Elucidation of the biosynthetic pathway for the production of the pigment chrysogine by *Penicillium chrysogenum*

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Running title: Chrysogine biosynthetic pathway in *Penicillium*

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ABSTRACT

Chrysogine is a yellow pigment produced by *Penicillium chrysogenum* and other filamentous fungi. Although it was first isolated in 1973, the biosynthetic pathway has so far not been resolved. Here, we show that the deletion of the highly expressed non-ribosomal peptide synthetase (NRPS) gene *Pc21g12630* (*chyA*) resulted in a loss in the production of chrysogine and thirteen related compounds in the culture broth of *P. chrysogenum*. Each of the genes of the *chyA*-containing gene cluster were individually deleted and corresponding mutants were examined by metabolic profiling in order to elucidate their function. The data suggest that the NRPS ChyA mediates the condensation of anthranilic acid and alanine into the intermediate 2-(2-aminopropanamido)benzoic acid, which was verified by feeding experiments of a *ΔchyA* strain with the chemically synthesized product. The remainder of the pathway is highly branched yielding at least thirteen chrysogine related compounds.

IMPORTANCE

Penicillium chrysogenum is used in industry for the production of β-lactams, but also produces several other secondary metabolites. The yellow pigment chrysogine is one of the most abundant metabolites in the culture broth next to β-lactams. Here, we have characterized the biosynthetic gene cluster involved in chrysogine production and elucidated a complex and highly branched biosynthetic pathway assigning each of the

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chrysogine cluster genes to biosynthetic steps and metabolic intermediates. The work further unlocks the metabolic potential of filamentous fungi and the complexity of secondary metabolite pathways.

INTRODUCTION

Penicillium chrysogenum and several other filamentous fungi produce the yellow pigment chrysogine (1, 2). Pigments are known to protect microorganisms against adverse environmental conditions, such as UV radiation, and often these compounds also exhibit antimicrobial activity (3). The function of chrysogine has not been extensively investigated, but unlike many other pigments it lacks antimicrobial or anticancer activity (4). *N*-pyruvoylanthranilamide (2-(2-oxopropanamido)benzamide), a related compound produced by *P. chrysogenum* (5) and also identified in *Colletotrichum lagenarium*, is equipped with anti auxin activity (6).

Chrysogine was first isolated in 1973 by Hikino et al. (5), who observed an increased production upon feeding with anthranilic acid and pyruvic acid. The putative biosynthetic gene cluster has been identified in *P. chrysogenum* (7, 8) and includes a non-ribosomal peptide synthetase (NRPS). Recently Wollenberg et al. showed that a dimodular NRPS is responsible for chrysogine biosynthesis in *Fusarium graminearum* and also suggested a putative cluster (9) homologous to the respective gene cluster of *P. chrysogenum*. However, the actual biosynthetic pathway has remained elusive.

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NRPSs are complex multi-modular enzymes that use amino acids and carboxylic acids as substrates (10). The genome of *P. chrysogenum* contains ten genes that encode NRPSs (11). Nonetheless, transcriptomic analysis performed on chemostat cultures of *P. chrysogenum* Wisconsin 54-1255 and the industrially improved DS17690 strain showed that only four of these *NRPS* genes are expressed (11). This set includes three *NRPS* genes that are respectively involved in the biosynthesis of penicillins (12), roquefortines (13) and hydrophobic cyclic tetrapeptides (14). The fourth highly expressed *NRPS* (7–9) is therefore potentially involved in the biosynthesis of chrysogine, that is among the most abundant secondary metabolites produced by this fungus. Furthermore, five genes flanking *Pc21g12630* are also highly co-expressed, suggesting they form a gene cluster (11).

Here, by overexpression and deletion of the core *NRPS* gene of the chrysogine pathway, deletion of the individual pathway gene and by feeding experiments using chemically synthesized intermediates, we elucidate a complex and branched pathway of at least thirteen compounds, assigning a function to each enzyme of the biosynthetic gene cluster.

RESULTS

Identification of chrysogine related compounds

In order to identify the secondary metabolites produced by the NRPS Pc21g12630, this gene was deleted from *P. chrysogenum* DS68530 by homologous recombination. In this strain, the penicillin cluster is removed (15, 16), facilitating further identification of other

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92 secondary metabolites as the metabolite profile is not dominated by beta lactams. The
93 strain deleted of the *Pc21g12630* gene did not produce chrysogine and thirteen other
94 metabolites, from now on referred to as chrysogine related compounds (Table 1). This
95 identified Pc21g12630 as the NRPS responsible for chrysogine biosynthesis and thus
96 this gene was named *chyA*.
97 Compounds **1**, **2**, **3**, **4**, **8** and **13** were isolated by preparative HPLC and their structures
98 were determined by NMR (Supplemental material). Compound **1** was confirmed to be
99 chrysogine and **3** was identified as *N*-pyruvoylanthranilamide (2-(2-
100 oxopropanamido)benzamide). These compounds were first described in *P.*
101 *chrysogenum* by Hikino et al. (5). **2** was found to be *N*-acetylalanylanthranilamide (2-(2-
102 acetamidopropanamido)benzamide), previously isolated from a marine *Penicillium*
103 species (17). **4**, **8** and **13** were identified as novel metabolites that are clearly related to
104 chrysogine. The structures of compounds **14** (2-(2-aminopropanamido)benzoic acid)
105 and **15** (the amidated form of compound **14**, 2-(2-aminopropanamido)benzamidine)
106 were further confirmed by the comparison of their HPLC retention time with those of the
107 independently synthesized standards (Supplemental material). The structures of
108 compounds **5** and **12** were proposed based on their molecular formula. We could not
109 assign a structure to **6**, **7**, **9** and **10** that could not be isolated due to their low
110 production.
111 Transcriptomic analysis performed on chemostat cultures of *P. chrysogenum* Wisconsin
112 54-1255 and the industrial improved DS17690 strain showed that five genes flanking
113 *chyA* (*Pc21g12570*, *Pc21g12590*, *Pc21g12600*, *Pc21g12610*, *Pc21g12620*) were also
114 highly expressed, indicating that they could be part of the chrysogine gene cluster (11)

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(Figure 1). Furthermore, quantitative PCR confirmed the expression of the above listed genes in the DS68530 strain after 48 h of growth in a SMP medium (Figure S2). Therefore, we tentatively assigned these as *chy* genes. *Pc21g12640*, found adjacent to the *chy* genes, exhibits a strong similarity with a cutinase transcription factor beta from *Fusarium solani* (11). Although not significantly expressed in DS68530, its possible role as regulator of the cluster was also investigated.

Expression of the *NRPS chyA* in a chrysogine cluster deleted strain

In order to identify the products of the *NRPS chyA*, a chrysogine cluster deleted strain (8) was used to overexpress the *chyA* gene from an episomal AMA1 based plasmid. The *chyA* overexpressing strain produced compounds **14**, **8** and **13** (Figure 2). It is likely that compound **14** is the immediate product of the NRPS and that this compound is derived from the condensation of anthranilic acid and alanine. **8** and **13** could respectively be derived from compound **14** by addition of a malonyl and glutaminyl group. Our data suggest an immediate branching of the pathway, where two groups of compounds are derived from **8** and **13**.

Metabolite profiles of *chy* gene deletion strains

The expression of *chyA* in a chrysogine cluster deleted strain allowed the identification of the product of the NRPS and metabolites produced early in the pathway. To elucidate how the initial products were further modified by the enzymes of the cluster and resolve the complete pathway, individual *chy* genes knockout strains were made and metabolite profiling was performed (Table 2).

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138 The deletion of *chyD* led to a depletion of most chrysogine related metabolites – only
139 compounds **14**, **8** and **13** were accumulated during cultivation of this mutant. This
140 suggests that ChyD is an early enzyme of the pathway, being responsible for converting
141 **14**, **8** and **13** into downstream compounds. Based on its formula, we propose that **14** is
142 converted into **15**, which is its amidated form.
143 The *ΔchyC* strain showed a metabolite profile similar to the *ΔchyD* strain suggesting
144 that ChyC could be also involved in the conversion of **14**, **8** and **13**. Nonetheless,
145 downstream compounds were still produced in low amount in the *ΔchyC* strain.
146 In the *ΔchyE* strain, **2**, **4**, **8** and **12** were not detected or produced in low concentrations
147 compared to the parental strain, suggesting that these compounds belong to the same
148 initial branch of the pathway. Based on the structures and molecular formula available,
149 **2**, **4** and **12** are derived from **8**, with **4** being most likely spontaneously converted into **2**
150 and **12**. Since ChyE affected the production of **8** and downstream compounds and
151 accumulated **14** after 96 h of growth, we propose that this enzyme converts **14** into **8**.
152 A trend opposite to the metabolite profile of *ΔchyE* can be observed in the *ΔchyM* strain.
153 Peak areas of **2**, **4**, **8** and **12** were comparable to DS68530 strain, while **1**, **3**, **7**, **9** and
154 **10** were absent or detected in low amounts. This indicates that these compounds are
155 part of an independent branch of the pathway and derived from **13**. The result is
156 confirmed by the accumulation of **14** and **13** in the *ΔchyM* strain. The molecular formula
157 of **5** suggests it is derived from **13** and that it is the precursor of **3**, which is further
158 converted into **1**, **7**, **9** and **10**. Because **3** and downstream compounds were not
159 produced in this mutant, we propose that ChyM is responsible for the conversion of **5**
160 into **3**. Chrysogine (**1**) is likely formed by a spontaneous ring closure from **3**.

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Compounds **9** and **10** are isomers, having the same molecular mass but different retention times on HPLC.

Finally, the *ΔchyH* strain showed a metabolite profile similar to that of *ΔchyM*, suggesting that both the enzymes are needed for the formation of the same compounds. Nonetheless, *ΔchyH* did not accumulate **13** and **5**, suggesting that ChyH forms **1**, **3**, **7**, **9** and **10** through an independent path. In the analysis of the mutant strains, we could not assign the position of compound **6** in the pathway. Based on the molecular formula, **6** could be an unstable precursor of **13**.

Metabolite profile and gene expression in a strain with a deletion of a putative transcription factor

Pc21g12640 encodes a putative transcription factor and, because of its chromosomal location in the vicinity of the chrysogine biosynthetic gene cluster, it would be plausible that it acts as a local regulator of this pathway. Although *Pc21g12640* is not significantly expressed in the DS68530 strain, transcription factors can regulate transcription even when present at very low levels. Therefore, to investigate its possible role as a regulator of the chrysogine cluster, *Pc21g12640* was deleted from strain DS68530. Nonetheless, the *ΔPc21g12640* strain did not show any significant changes in the chrysogine related metabolite profiles compared to the parental strain (Table 2). Similarly, qPCR indicated that the deletion of *Pc21g12640* did not significantly affect the expression of the genes of the chrysogine cluster (Figure S2). Thus, *Pc21g12640* is not part of the chrysogine biosynthetic gene cluster.

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Feeding of the *ΔchyA* strain with compounds 14 and 15

In order to further investigate the role of compounds **14** and **15** as potential NRPS products, the *ΔchyA* strain was fed with chemically synthesized variants of these. Based on the formula, **15** is the amidated form of **14**.

Above we showed that the expression of *chyA* in the chrysogine deleted strain resulted in the production of **14**, **8** and **13**. The *ΔchyA* strain fed with **14** produced **2**, **4** and **8**, while **13** and downstream compounds were not detected (Figure 3A). This result suggests that the conversion of **14** into **8** is faster than its conversion into **13**. The feeding with **15** resulted in the production of metabolites that are derived from **8** (**2**, **4**, **12**) and **13** (**1**, Figure 3B). As compound **15** is very similar to compound **14**, we suggest that **15** undergoes the same reactions, being converted into **4** by ChyE and into **5** by a transaminase. Since *ΔchyH* affected the production of **3** and downstream metabolites without any accumulation of **5**, we propose that ChyH is involved in the biosynthesis of **3** from **15**. Therefore, the late metabolites can be formed from two different paths.

Distribution and diversity of chrysogine gene clusters in *Penicillia* species

Since the above studies characterized the chrysogine biosynthetic gene cluster, the distribution of this gene cluster in other *Penicillia* species was investigated (Figure 1). The *chy* genes and *Pc21g12640* from *P. chrysogenum* were blasted against the genomes of two known chrysogine producers (2), *P. nalgiovense* and *P. flavigenum*, recently sequenced by Nielsen et al. (18). These genomes contain a chrysogine gene cluster with similar gene organization, while a *Pc21g12580* homolog is missing, supporting the notion that this gene is not essential for chrysogine biosynthesis.

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207 Interestingly, *P. flavigenum* has two extra genes nearby the *NRPS* gene, suggesting
208 that it may produce additional chrysogine related metabolites.
209
210
211 **DISCUSSION**
212
213 Chrysogine was isolated from the culture broth of *P. chrysogenum* in 1973 (5) and
214 found to be produced also by other filamentous fungi (1, 2). Chrysogine biosynthesis is
215 mediated by a dimodular NRPS that we recently identified in *P. chrysogenum* (7, 8)
216 and that was also shown to be responsible for chrysogine biosynthesis in *Fusarium*
217 *graminearum* (9). Although the biosynthetic gene cluster was suggested, the role of
218 the enzymes in the pathway has sofar not been characterized. In this work, we
219 assigned a function to each enzyme of the cluster and elucidated a complex pathway,
220 validating the compound structures by NMR. The pathway is highly branched, with
221 some enzymes involved in multiple steps of the biosynthesis (Figure 1).
222 The NRPS ChyA is a 260 kDa dimodular enzyme which is predicted to contain two
223 adenylation domains. The increased production of chrysogine upon feeding with
224 anthranilic acid and pyruvic acid (5) suggests these molecules are possible substrates
225 of the NRPS. However, here we identify compound **14** as the direct product of ChyA,
226 showing that the NRPS in addition to anthranilic acid utilizes alanine instead of pyruvic
227 acid. However, alanine is readily derived from pyruvic acid by transamination which
228 explains why pyruvic acid stimulates chrysogine production. NRPSsp, NRPSpredictor2
229 and SEQL-NRPS (19–21) were used for predicting the substrates of ChyA, but results

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were inconclusive. For the first adenylation domain, phenylalanine, a hydrophobic aliphatic amino acid, 2,3-dihydroxy-benzoic acid or salicylic acid was predicted, while for the second adenylation domain proline or a hydrophobic aliphatic amino acid was suggested. This shows that with fungal NRPS, predictions can be unreliable necessitating experimental validation. Compound **14** acts as a substrate for several enzymes, which immediately results in a split in the pathway by forming **8**, **13** and **15**, the latter being the amidated form of compound **14**. Two independent groups of compounds are derived from **8** and **13**. Since **15** undergoes the same reactions as **14**, the more distal metabolites in the pathway can be formed via either branch that converge.

Transcriptomic data (11) suggested that *chyA* and five flanking genes could form a cluster. These genes are co-expressed under a set of conditions, whereas expression profiles in the flanking regions of the putative gene cluster vary. Metabolic profiling of the mutant strain indicated that ChyE is a malonyl transferase, which can convert **14** and **15** into **8** and **4**, respectively. Interestingly, the expression of *chyA* in a chrysogine cluster deleted strain showed that **14** can be converted into **8** without involvement of any of the enzymes of the cluster; this conversion likely involves a transferase. In line with this observation, the deletion of *chyE* did not lead to a complete depletion of **8** and downstream metabolites, although it significantly decreased the amounts produced. These data suggest that *chyE* is part of the biosynthetic cluster, as it is co-expressed together with the other genes (11) and its deletion affects chrysogine metabolites production, but one or more other transferases can catalyze the same reactions. The

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orthologous gene in *Fusarium* species is not involved in chrysogine biosynthesis, showing a different expression pattern compared to the genes of the cluster (9).

Also compound **13** was formed by the strain that solely expresses *chyA*, likely through the involvement of a transaminase, which is not part of the gene cluster. Based on sequence alignment, no genes encoding for a transaminase have been identified in the immediate vicinity of the chrysogine genes, but the genome contains many transaminases.

Our data indicate that ChyD is an amidase, being responsible for the amidation of the carboxylic acid moiety of **14**, **8** and **13**, in line with the bioinformatics prediction of ChyD as an asparagine synthetase, which amidates aspartate to form asparagine. The *ΔchyC* strain showed a metabolite profile similar to that of the *ΔchyD* strain, suggesting that ChyC is involved in the same reactions as ChyD. Indeed, downstream compounds were still produced in low amount in the *ΔchyC* strain. For this reason, we speculate that ChyC plays a more minor role in the amidation reactions compared to ChyD, whose deletion abolished completely the production of the late metabolites. Protein alignment does not provide sufficient information to assign a specific function to ChyC. ChyH and ChyM are predicted to be involved in oxidation reactions and form compound **3** from **15** and **5,** respectively. **3** originates from two further branches in the pathways, yielding chrysogine and **7**, **9** and **10**.

Regulatory genes are usually clustered with secondary metabolite biosynthetic genes (22). Therefore, we hypothesized that the putative transcription factor Pc21g12640 can regulate the expression of the chrysogine genes, since *Pc21g12640* is located downstream of *chyA*. Nonetheless, metabolite profiling and qPCR of the deletion strain

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gave no indications that Pc21g12640 is involved in the regulation of the *chy* genes. This conclusion is supported by the absence of the transcription factor in *Fusarium* and the other filamentous fungi investigated by Wollenberg et al. (9), although the orthologous gene is present in the genome of other *Penicillia* species (Figure 1).

As already shown for some other fungal secondary metabolites clusters (22, 23), it is possible that the chrysogine biosynthetic genes are regulated by other transcription factors. Moreover, epigenetic regulation has been suggested for the chrysogine cluster. Shwab et al. (24) first demonstrated that secondary metabolites genes can be regulated by chromatin remodeling, for example by histone acetylation. In *P. chrysogenum* DS68530, the deletion of the histone deacetylase *hdaA* resulted in a significant downregulation of the *chy* genes expression and subsequent reduction of chrysogine biosynthesis (Guzman, Salo and Samol, unpublished data).

Secondary metabolite pathways can provide a wide range of compounds from the initial scaffold molecule. Moreover, the same compounds can be produced through different paths. Branched secondary metabolite pathways have been described before in *P. chrysogenum* (13). The chrysogine pathway is even more branched than the previously described roquefortine pathway, and in this case, chrysogine is the final product of one ramification. As a pigment, chrysogine could contribute to protect the cell from UV light. No antimicrobial activity has been found for this metabolite (4) nor for *N*-acetylalanylanthranilamide (**2**), which was also identified in a marine fungus (17). The function of the other metabolites in the cell remains unknown. Nonetheless, the approaches used in this work and the established methods can provide a blueprint for the elucidation of novel secondary metabolite pathways that potentially specify

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298 unknown bioactive compounds. Moreover, the understanding of the biosynthetic
299 mechanisms can help to develop new molecules by feeding with chemically modified
300 intermediates.
301
302
303 **MATERIALS AND METHODS**
304
305 **Fungal strains, media and culture conditions**
306 *P. chrysogenum* DS68530 was kindly provided by DSM Sinochem Pharmaceuticals.
307 DS68530 lacks the penicillin gene cluster and the *hdfA* gene (15, 16). For RNA
308 extraction and metabolite analysis, strains were pre-grown in YGG medium (25) for 24
309 hours. Next, 3 ml of culture inoculum was transferred into 22 ml of secondary
310 metabolites production (SMP) medium (13) and growth was continued for the time
311 indicated. The *Pc21g12630* (*chyA*) overexpression strain was grown in SMP medium,
312 lacking urea and CH_3COONH_4, and supplemented with 2 g/L acetamide for plasmid
313 maintenance. The *ΔchyA* strain was fed with 300 µM of compound **A** or **B** after 48 h of
314 growth. All cultivations were performed as 25 ml cultures in 100 ml erlenmeyer flasks
315 shaken at 200 rpm and 25°C.
316
317 **Construction of deletion and overexpression plasmids**
318 Plasmids for the deletion of the chrysogine genes were built by PCR amplification of 1 -
319 2 kbp of the 5' and 3' flanking regions of each gene, using gDNA from the DS68530

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strain as template. All primers used in this study are listed in Tables 3 and 4, the constructed plasmids are shown in the supplementary material.

For the deletion of *Pc21g12630 (chyA)*, *Pc21g12570* (*chyE*), *Pc21g12590* (*chyH*), *Pc21g12610* (*chyM*) and *Pc21g12640* genes, the Multisite Gateway® Three-Fragment Vector Construction Kit (Invitrogen) was used. PCR products were inserted into the donor vectors pDONR4-R1 and pDONR2-R3 by the BP clonase II™ reaction. The resulting plasmids were mixed with the vector carrying the selection marker (pDONR-amdS or pDONR-phleo), the destination vector pDESTR4-R3 and the LB clonase II™ mixture, to form the final constructs. The acetamidase gene *amdS* (25, 26) was employed as a marker for the deletion of *chyH, chyM, Pc21g12640* genes, while the phleomycin resistance gene was used for selecting *chyA* and *chyE* deleted strains. The modular cloning (MoClo) system (27) was used for building *Pc21g12600* (*chyC*) and *Pc21g12620* (*chyD*) deletion vectors containing an *amdS* marker cassette.

Due to its strength, the *pcbC* promoter was chosen for overexpression of *chyA*, followed by the *penDE* terminator. All genetic elements were amplified from *P. chrysogenum* DS68530 gDNA and the *chyA* expression cassette was built in subsequent steps of digestions and ligation, using pCM251 (Euroscarf) as backbone vector. The promoter and terminator were digested with *BamHI*, *PmeI* and *NotI* enzymes for cloning into pCM251. *ChyA* was inserted into the resulting pCM251 plasmid after digestion with *AscI* and *PmeI*. The expression cassette was digested with *NotI* for the insertion into pDSM-JAK108 (28), to form pDSM108_AV1. pDSM-JAK108 contains the AMA1 (autonomous maintenance in *Aspergillus*) (29) sequence, the *dsRed* gene for visualization of the cells and the essential gene *tif35*. In this study the *tif35* gene on the plasmid was replaced

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with an *amdS* cassette by *in vivo* homologous recombination in *P. chrysogenum*. The *amdS* cassette containing 100 bp flanks homologous to pDSM108_AV1 was obtained by oligonucleotide extension-PCR, using pDONR-amdS as template.

Transformation and purification procedures

The deletion plasmids (1.5 µg) were linearized and transformed into *P. chrysogenum* DS68530 protoplasts using a standard protocol (30). pDSM108_AV1 (1 µg) was linearized by digestion with *MluI* enzyme and co-transformed with the *amdS* cassette (1 µg). The transformants were plated on respective selective media (T-agar) (25) and grown at 25°C for 5 days. For strain purification, the colonies were transferred to minimal selective solid media (S-agar) and sporulation media (R-agar) (25). Rice batches were prepared for inoculation of conidia and long-term storage.

Analysis of the gene deletion strains

The absence of the deleted genes was verified by PCR, with gDNA isolated from the knockout strains after 48 h of growth, using an adapted yeast gDNA extraction protocol (31). Primers binding outside the homologous flanking regions were used for amplification of the targeted fragment, after which the PCR products were further verified by sequencing (Macrogen, UK). To verify the correct integration of the *amdS* cassette into pDSM108-AV1, colony PCR were performed on red colonies (bearing the AMA1 plasmid as seen by the DsRed marker on the plasmid).

RNA extraction, cDNA amplification and qPCR analysis

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366 Total RNA was isolated from the DS68530 and *ΔPc21g12640* strains after 48 h of
367 growth in SMP medium, by using the Trizol™ (Invitrogen) extraction method with
368 additional DNAse treatment (Turbo DNA-free™ kit, Ambion). For the cDNA synthesis,
369 500 ng of RNA were used (iScript™ cDNA synthesis kit, Bio-Rad). The γ-actin gene
370 was used for normalization. The expression levels were measured in technical
371 duplicates with a MiniOpticon™ system (Bio-Rad) using the Bio-Rad CFX™ manager
372 software, which determines the threshold cycle (Ct) values automatically by regression.
373 The SensiMix™ SYBR Hi-ROX kit (Bioline) was used as mastermix for qPCR. The
374 reactions were run as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec,
375 55 °C for 30 sec and 72°C for 30 sec.
376
377 **Metabolite profiling**
378 All the strains used were grown in triplicates for metabolite analysis. Samples were
379 collected after 48 h from the *chyA* overexpression strain and after 48 and 96 h from the
380 deletion mutants and the parental strain. Samples were taken before the feeding of
381 *ΔchyA*, immediately after the feeding and then after 48 h. All the samples from the
382 different experiments were centrifuged for 10 min, after which the supernatant was
383 filtered with 0.2 µm polytetrafluorethylene (PTFE) syringe filters and stored at -80°C.
384 The analysis of secondary metabolites was performed with an Accella1250™ HPLC
385 system coupled with the ES-MS Orbitrap Exactive™ (Thermo Fisher Scientific, CA),
386 following the method described by Salo *et al.* (32).
387
388

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480 Chapter 1 Fungal ABC Transporter Deletion and Localization Analysis. Methods

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LEGENDS TO THE FIGURES

Figure 1 Representation of the chrysogine biosynthetic gene cluster and proposed pathway. The chrysogine biosynthetic gene cluster in *P. chrysogenum* and

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two other chrysogine producing species. Genes with same color have >80% identity. This study identified ChyA as the NRPS, ChyE as malonyl transferase and ChyD as amidase; ChyC participates in amidation reactions, while ChyH and ChyM are involved in oxidation reactions. The substrates of ChyA and the compounds identified in this study are depicted in black, the putative structures and uncharacterized compounds are represented in red.

Figure 2 Chromatogram of culture broth from the *chyA* expressing strain. Total ion chromatogram (TIC, black) and extracted ion chromatograms (EIC, colored) of secondary metabolites produced by the *chyA* expressing strain after 48 h of growth in a SMP medium.

Figure 3 Chromatogram of culture broth from *ΔchyA* strain fed with 14 or 15. TIC (black) and EIC (colored) of secondary metabolites produced by the *ΔchyA* strain fed with **14** (A) or **15** (B) after 48 h from the feeding.

Table 1 Production of chrysogine and related metabolites from DS68530 strain. Numbers represent the peak areas of the compounds corrected for the internal standard reserpine. The culture broth of DS68530 strain was analyzed after 48 and 96 h of growth in a SMP medium.

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526 **Table 2 Secondary metabolites of the chrysogine pathway in the knockout**
527 **strains compared to the parental strain.** Numbers represent the peak areas of the
528 compounds corrected for the internal standard reserpine and relative to the parental
529 strain DS68530. The culture broth of the strains was analyzed after 48 and 96 h of
530 growth in a SMP medium.
531
532 **Table 3** Oligonucleotide primers used for amplifying the 5' and 3' flanking regions of the
533 targeted genes and for qPCR.

534 **Table 4** Oligonucleotide primers for amplification of P*pcbC*, *chyA* and T*penDE* for
535 cloning into pDSM-JAK108; amplification of *amdS* cassette for *in vivo* homologous
536 recombination into pDSM108_AV1; check the correct integration of *amdS* cassette into
537 pDSM108_AV1; check the absence of the genes in the knockout strains and
538 amplification of the deletion cassettes into the genome. PCR products were sent for
539 sequencing by using primers *phleo_seq* and *amdS_seq*, in order to check the purity of
540 the strains.
541

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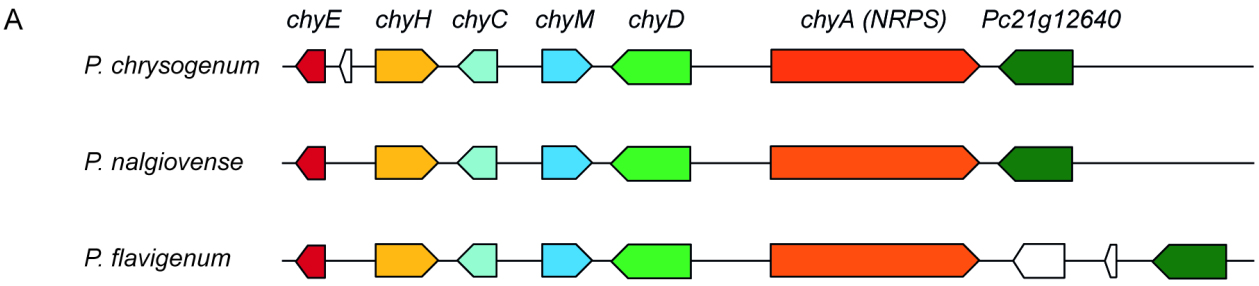

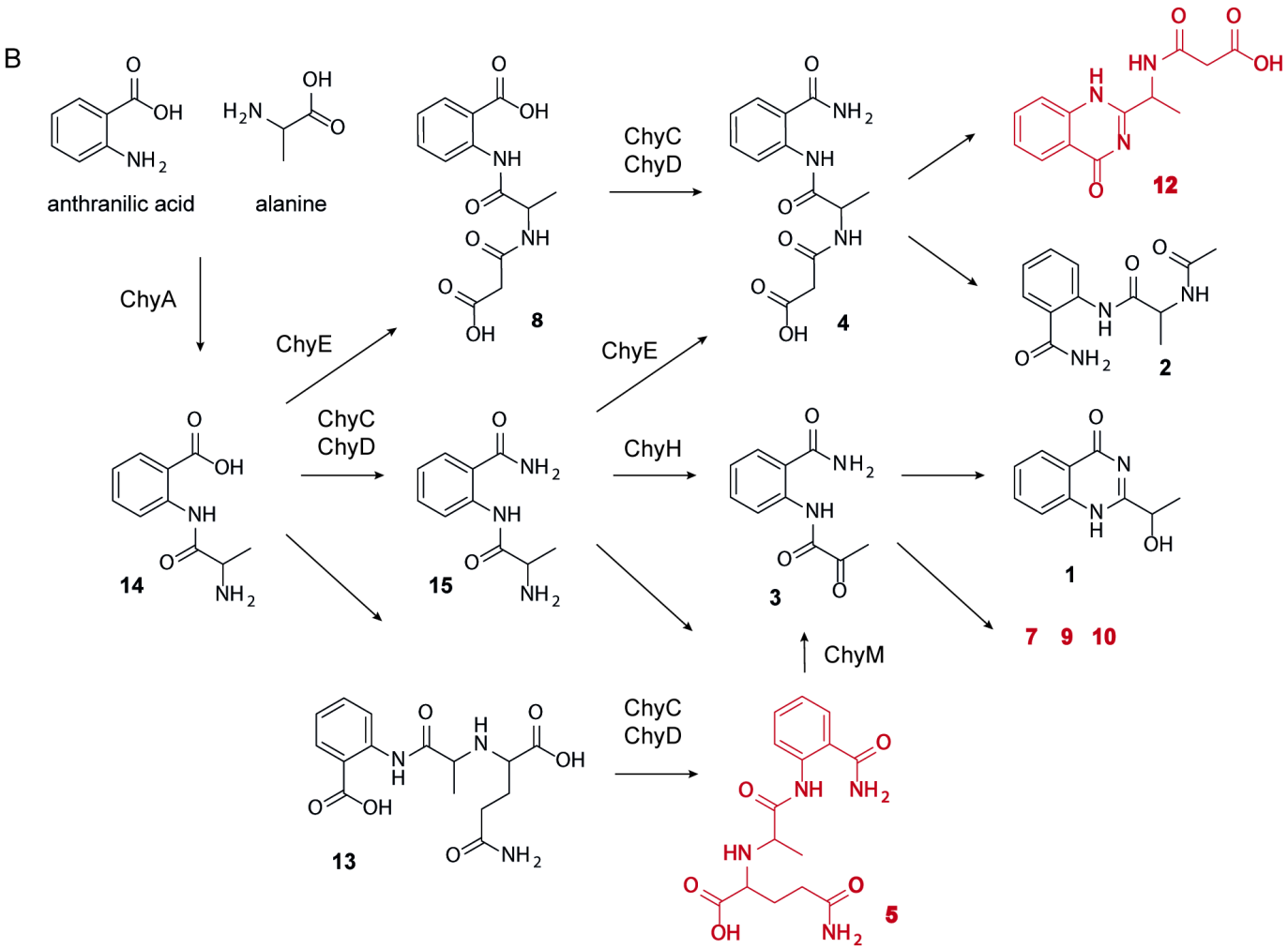

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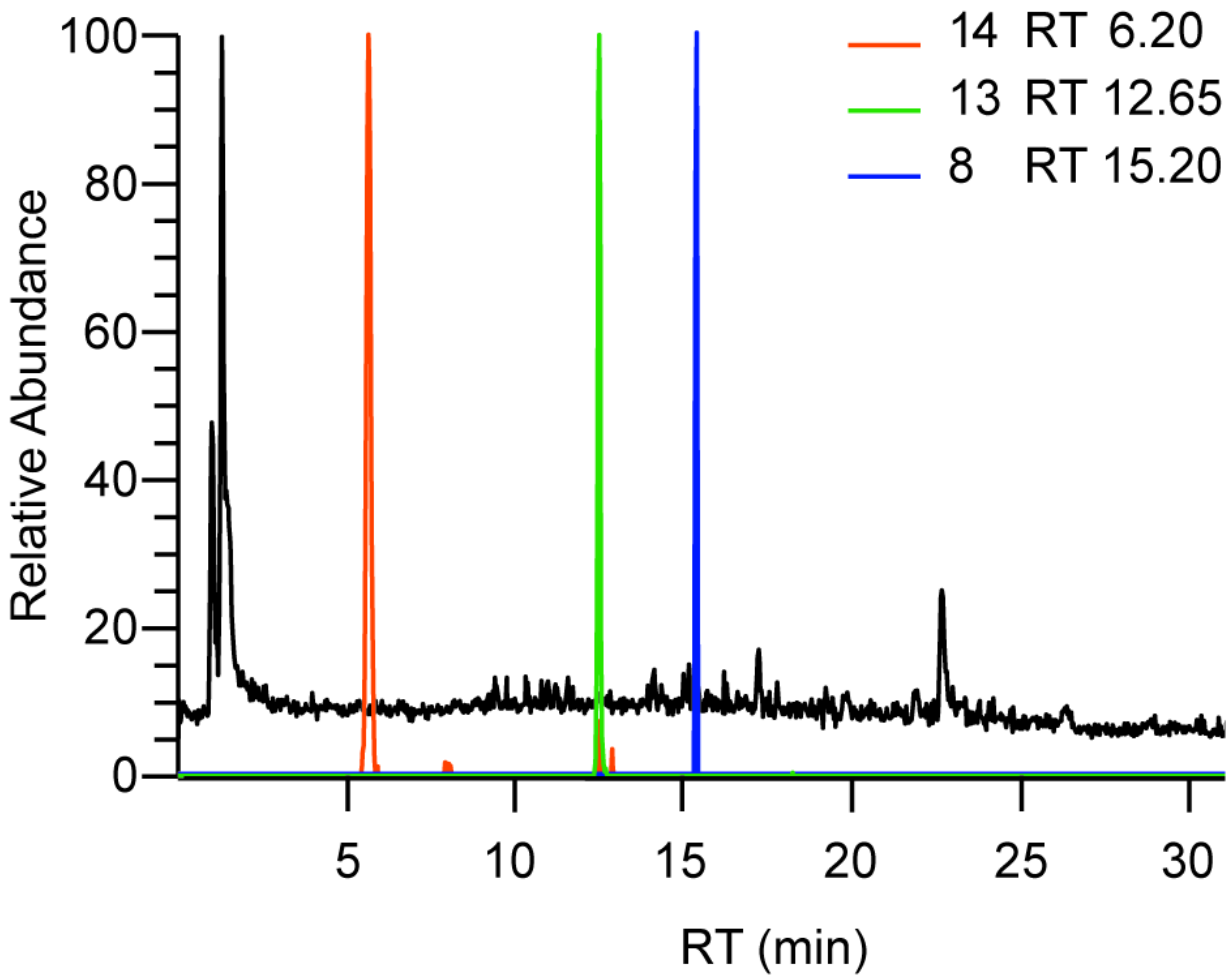

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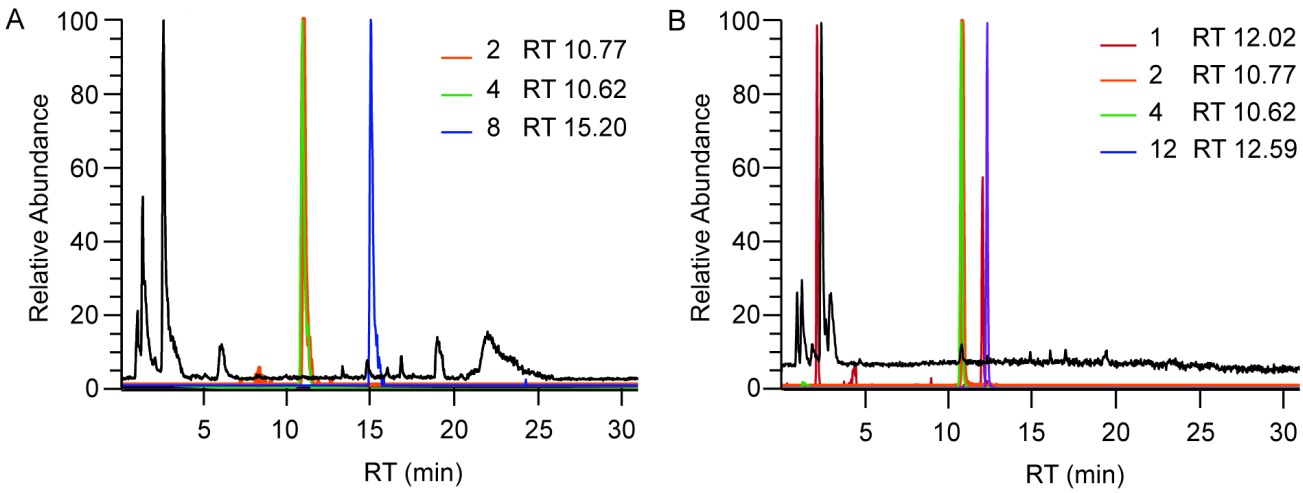

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compound number	compound name	formula	acquired [M+H]+	RT (min)	DS68530 48 h	DS68530 96 h
1	chrysogine	$C_{10}H_{10}O_2N_2$	191.08	12.02	185,02	236,37
2	2-(2-acetamidopropanamido)benzamide	$C_{12}H_{15}O_3N_3$	250.12	10.77	19,85	53,92
3	2-(2-oxopropanamido)benzamide	$C_{10}H_{10}O_3N_2$	207.08	10.32	0,65	11,26
4	3-((1-((2-carbamoylphenyl)amino)-1-oxopropan-2-yl)amino)-3-oxopropanoic acid	$C_{13}H_{15}O_5N_3$	294.11	10.62	24,84	152,64
5	(1-((2-carbamoylphenyl)amino)-1-oxopropan-2-yl)glutamine	$C_{15}H_{20}O_5N_4$	337.15	8.60	4,92	0
6	chrysogine related	$C_{15}H_{18}O_6N_3$	336.12	9.02	0	2,44
7	chrysogine related	$C_{13}H_{12}O_5N_2$	277.08	11.06	0	0,82
8	2-(2-(2-carboxyacetamido)propanamido)benzoic acid	$C_{13}H_{14}O_6N_2$	295.09	15.20	0	1,12
9	chrysogine related	$C_{20}H_{20}O_6N_4$	413.14	14.95	0,42	0,68
10	chrysogine related	$C_{20}H_{20}O_6N_4$	413.14	15.70	0,42	0,57
12	3-oxo-3-((1-(4-oxo-1,4-dihydroquinazolin-2-yl)ethyl)amino)propanoic acid	$C_{13}H_{13}O_4N_3$	276.10	12.59	0,50	1,10
13	2-(2-((4-amino-1-carboxy-4-oxobutyl)amino)propanamido)benzoic acid	$C_{15}H_{19}O_6N_3$	338.13	12.65	2,91	0,20
14	2-(2-aminopropanamido)benzoic acid	$C_{10}H_{12}O_3N_2$	209.09	6.20	1,15	0,18
15	2-(2-aminopropanamido)benzamide	$C_{10}H_{13}O_2N_3$	208.11	2.47	0,17	0

Max

Response ratio

Min

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	ΔchyC		*ΔchyD*		*ΔchyE*		*ΔchyH*		*ΔchyM*		*ΔPc21g12640*	
compound name	**48 h**	**96 h**	**48 h**	**96 h**	**48 h**	**96 h**	**48 h**	**96 h**	**48 h**	**96 h**	**48 h**	**96 h**
1	0,19	0,37	0	0	0,66	0,73	0,03	0,03	0,01	0,02	0,96	0,83
2	0,16	0,23	0	0	0	0,01	0,86	0,77	1,88	1,89	0,80	0,70
3	0,23	0,21	0	0	0,68	0,58	0	0,02	0	0	0,93	0,82
4	0,19	0,45	0	0	0	0,01	0,84	0,75	1,16	1,31	0,84	0,75
5	0	0	0	0	0,67	0,41	0,66	0	1,44	2,40	0,75	0,04
6	0	0,05	0	0	0	1,03	0	0,51	0	0,42	0	0,58
7	0	0,45	0	0,09	0	0,37	0	0	0	0	0	0,69
8	0	3,86	0,31	48,40	0	0,23	0	1,80	0	1,09	0	0,42
9	0	0	0	0	0,51	0,54	0	0	0	0	0,86	0,50
10	0	0	0	0	0,51	0,92	0	0	0	0	0,86	0,80
12	0,17	0,24	0	0	0	0	0,97	0,48	3,39	3,07	0,93	0,78
13	0	4,09	15,59	12,75	0,52	9,16	0,67	0,22	1,36	40,26	0,81	2,86
14	0,33	15,87	11,56	16,73	0,34	2,34	0,92	0,28	1,98	13,99	0,89	1,24
15	0	0	0	0	0,05	0,01	0,99	0	2,64	0,05	0,69	0

Max

Response ratio

Min

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chyA_5'_fw	GGGGACAACTTTGTATAGAAAAGTTGGGTACCGTTCGTACACACCATTCCGGCTG
chyA_5'_rv	GGGGACTGCTTTTTTGTACAAACTTGCATCGATCCTTGATGCCTACAGC
chyA_3'_fw	GGGGACAGCTTTCTTGTACAAAGTGGAAGAGATTGCGAGAGTTGGCTGG
chyA_3'_rv	GGGGACAACTTTGTATAATAAAGTTGGGTACCACTCGAAGGCTCCGTTCTCGGC
chyC_5'_fw	TTGAAGACAATGCCCCTGCAGGTGGGTCGGTATCACAACGACCG
chyC_5'_rv	TTGAAGACAATTGCGTCCCGTTCGCATGGTTACATAGCT
chyC_3'_fw	GGGGACAACTTTGTATAATAAAGTTGGGTACCACTCGAAGGCTCCGTTCTCGGC
chyC_3'_rv	TTGAAGACAAACTAGTTGAAGAAGTTGGTGTAGTTTGAGAATG
chyD_5'_fw	TTGAAGACAAGGAGCCTGCAGGGATCTCAAAGACTATTATCAAGGAAAGGA
chyD_5'_rv	TTGAAGACAAAGCGGGGGTGTCGCATGATTATATCTATAGT
chyD_3'_fw	TTGAAGACAAGGAGTTTGAGATTGAGATGAAAGGATTTGGAAAG
chyD_3'_rv	TTGAAGACAAAGCGCCTGCAGGCGGGCATCTTCACGATCCAATAG
chyE_5'_fw	GGGGACAACTTTGTATAGAAAAGTTGCGTGCAGCAAAGACGACATTCG
chyE_5'_rv	GGGGACTGCTTTTTTGTACAAACTTGAGGTATTGGGAATAGACCGGCC
chyE_3'_fw	GGGGACAGCTTTCTTGTACAAAGTGGCAGTATATCTGACGAGGAAGTGGG
chyE_3'_rv	GGGGACAACTTTGTATAATAAAGTTGTCTCCTAGTATCCGACTTCTCCG
chyH_5'_fw	GGGGACAACTTTGTATAGAAAAGTTGGCATCGTAATATGCTCGATTTGG
chyH_5'_rv	GGGGACTGCTTTTTTGTACAAACTTGAGTCTATATAAGCGCTCGGAGGC
chyH_3'_fw	GGGGACAGCTTTCTTGTACAAAGTGGATGAGAGTGAAAGTGTTCAGTGCG
chyH_3'_rv	GGGGACAACTTTGTATAATAAAGTTGGAAGGACCCCTGAGACAGAACC
chyM_5'_fw	GGGGACAACTTTGTATAGAAAAGTTGAACTTCGAGTCGCAGTATGCGG
chyM_5'_rv	GGGGACTGCTTTTTTGTACAAACTTGGGTGTAATGGAACCCATTGCAAGG
chyM_3'_fw	GGGGACAACTTTGTATAGAAAAGTTGAACTTCGAGTCGCAGTATGCGG
chyM_3'_rv	GGGGACTGCTTTTTTGTACAAACTTGGGTGTAATGGAACCCATTGCAAGG
Pc21g12640_5'_fw	GGGGACAACTTTGTATAGAAAAGTTGCAAGAGATTGCCGATAACATTGTGG
Pc21g12640_5'_rv	GGGGACTGCTTTTTTGTACAAACTTGATGACTGGTCCGAGGTACTGG
Pc21g12640_3'_fw	GGGGACAGCTTTCTTGTACAAAGTGGATCATGCACGATGTGGTCATATGG
Pc21g12640_3'_rv	GGGGACAACTTTGTATAATAAAGTTGGCGGCCGCAGATTTCTCGACGTCCGATC
chyA_qPCR_fw	GCACAGGCCAAAGTAACACGTCC
chyA_qPCR_rv	CCGAGGGTTTGTGGTGGATGCC
chyC_qPCR_fw	GTAGACGCCGGTGAGACTTTGATCG
chyC_qPCR_rv	CAACCTAAGCGTCTAATTTTCATCGC
chyD_qPCR_fw	GGAATTCGCTGGCTAACTGGTCTCG
chyD_qPCR_rv	GGCATGTGGTAGACGAATTGGAGC
chyE_qPCR_fw	GGCAAGGGAAATGAATCCAGGTGGC
chyE_qPCR_rv	GATAGATGCCGCTTGTTCGGACC
chyH_qPCR_fw	GGTTGTGGAGCTCTACGAGGCTG
chyH_qPCR_rv	CTGGCAGGGCTCGTCGGTC
chyM_qPCR_fw	CCTGCATGCAGCTCCATACGAGC
chyM_qPCR_rv	CCAACAATAGGTGGAAACAGCTCAGAC
Pc21g12640_qPCR_fw	TGTCTCTCTGTGGGCTGTTCTCAG
Pc21g12640_qPCR_rv	CAAGAGTTCTTACGATGCGTGGCTG
actin_qPCR_fw	CGACTACCTGATGAAGATCCTCGC
actin_qPCR_rv	GTTGAAGGTGGTGACGTGGATACC

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Primer name	Sequence (5'-3')
P*pcbC_fw*	CAGTGGATCCACGCGTGTCTGTCAATGACCAATAATTGG
P*pcbC_rv*	CATGGTTTAAACGGCGCGCCGGTGTCTAGAAAAATAATGGTGAAAAC
chyA_cloning_fw	CATGGGCGCGCCATGGCTGCCCCATCCATATCGC
chyA_cloning_rv	CATGGTTTAAACTTACTCGAGATATTCGCAGACTGTCTCTTC
T*penDE_fw*	TCTGCGAATATCTCGAGTAAGTTTAAACCAATGCATCTTTTGTATGTAGCTTC
T*penDE_rv*	TCACTATAGGGCGAATTGGAGCTCCACCGCGGTGGCGGCCGCGGCCGCTGATATCCTGTC TTCAGTCTTAAGAC
amdS_hom_rec_fw	CTTATTAATTTGATGTAGGTAAGCCCGCCACAAATATATATTTTTACAAGATACCGTGGAAAA ACTTCGTGCTATCACAAAACAGTATACAAAAAATAAGTGGATCCCCCGGGCTGCAGG
amdS_hom_rec_fw	TCCCCTCGAGCTTGTCTGTGATTGCGTTTTTTCTAACACTTGTTGTTGCATCCGATCCGTCCC TACCAATTATTGGTCATTGACAGACACGCGTACCGCTCGTACCATGGGTTGAGTGGT
amdS_int_fw	ACAGCGGAAGACAAGCTTCTAATAAGTGTCAGATAGCAAT
amdS_int_rv	GTTGGCTCCCAGAGCAGCGGTGTCTTTCGTATTCAGGCAGCTAAAC
chyA_fw	CCATATCGCCGTTATTTGCC
chyA_rv	GACGGCAACATGTAGGAAAC
chyC_fw	ATGGCCCGCATCCTGATCAC
chyC_rv	TTAAGCTGGGAGCTTAATACCGGTGAT
chyD_fw	ATGTGTGGAATAAGTGCATTTCTGTGTC
chyD_rv	TCAGTTTGGCAGGGCACCAG
chyE_fw	ATGGACTCAGTGAGCAATCTAAAG
chyE_rv	CTATTCTGACAGCCACTGCAAA
chyH_fw	TCGCGATGCCGACTATAAAG
chyH_rv	GCCCATAGAAGCTGAACATC
chyM_fw	ATGGGTTCCATTACACCCTCGC
chyM_rv	TCACCAGAATGCTGCACACCG
Pc21g12640_fw	ATGTCTTCAGCCCCCGGTCT
Pc21g12640_rv	CTAGAATATGTCATCCTCGGATTGGAACC
actin_fw	ATGGAGGGTATGTTATTCCAGTTGTGG
actin_rv	TGCGGTGAACGATGGAAGGACC
phleo in chyA locus_fw	CAACGCCCACGAGCATCTGGT
phleo in chyA locus_rv	GCCAGAAACTCGACTCGTGGCTC
amdS in chyC locus_fw	TCACCAGAATGCTGCACACCG
amdS in chyC locus_rv	GATACCCCTTAGCCCGTCATCCAAA
phleo in chyE locus_fw	CCATGTCGGGTGTAGATCG
phleo in chyE locus_rv	GCCCATAGAAGCTGAACATC
amdS in chyM locus_fw	CTTGTCAAGTCTGCGACCAGCAC
amdS in chyM locus_rv	ACGAAGAGGCACTCGCGTCAC
amdS in Pc21g12640 locus_fw	CAAACAGATGAAGACTGGGG
amdS in Pc21g12640 locus_rv	GGCTCAAACTTGCGCTTAG
phleo_seq	ATGGCCAAGTTGACCAGTGCCGTT
amds_seq	TCCCCTAAGTAAGTACTTTGCTA

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