Post-translational enzyme modification by the phosphopantetheinylation transferase is required for lysine and penicillin biosynthesis but not for roquefortine or fatty acid formation in Penicillium chrysogenum
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Post-translational enzyme modification by the phosphopantetheinyl transferase is required for lysine and penicillin biosynthesis but not for roquefortine or fatty acid formation in *Penicillium chrysogenum*.
Nonribosomal peptide synthetases (NRPSs) and polyketide synthases (PKSs) require a post-translational phosphopantetheinylation to become active. This reaction is catalysed by the 4'-phosphopantetheinyl transferase (PPTase). The ppt gene of *Penicillium chrysogenum*, encoding a protein that shares 50% homology with the stand-alone large PPTases, has been cloned. This gene is present as a single copy in the genome of the wild type and high penicillin producing strains (containing multiple copies of the penicillin gene cluster). Amplification of the *ppt* gene produced increases in isopenicillin N and benzylpenicillin biosynthesis. A PPTase defective mutant (Wis54-PPT−) was obtained. It required lysine and lacked pigment and penicillin production, but it still synthesized normal levels of roquefortine. The biosynthesis of roquefortine does not appear to involve a PPTase-mediated modification of the synthesizing enzymes. The PPT− mutant did not require fatty acids, which indicates that activation of the fatty acid synthase occurs by a different PPTase. Complementation of Wis54-PPT− with the *ppt* gene, restored lysine biosynthesis, pigmentation and penicillin production, which evidences the wide range of processes controlled by this gene.

Keywords: 4'-phosphopantetheinyl transferase, lysine, beta-lactam antibiotics, post-translational modification, roquefortine, secondary metabolism

Abbreviations used: ACPS, acyl carrier protein synthase; ACV, δ(L-α-amino acidyl)-L-cysteinyl-D-valine; ACVS, δ(α-amino acidyl)-cysteinyl-valine synthetase; ble, bleomycin and phleomycin resistance gene; CP, complex penicillin production medium; DP, defined production medium; FAS, fatty acid synthase; GATase, glutamine amidotransferase; HPLC, high performance liquid chromatography; IOD, integrated optical density; LB, Luria- Bertani broth medium; NRPS, nonribosomal peptide synthetase; PKS, polyketide synthases; ORF, open reading frame; Pgdh, glutamate dehydrogenase gene promoter; PPTase, 4'-phosphopantetheinyl transferase; RT-PCR, reverse transcription-PCR; Teyc1, cytochrome c1 transcriptional terminator.
INTRODUCTION

*Penicillium chrysogenum* is a filamentous fungus which produces penicillins with an aromatic side chain, like benzylpenicillin or phenoxymethylpenicillin [1]. The pathway for penicillin formation has been largely elucidated [2]. It starts with the non-ribosomal biosynthesis of the tripeptide \( \delta(L-\alpha\text{-aminoacidyl})-L\text{-cysteinyl-D-valine} \) (ACV), synthesized by the large multienzyme \( \delta(\alpha\text{-aminoacidyl})\text{-cysteinyl-valine synthetase (ACVS)} \), a typical non-ribosomal peptide synthetase (NRPS) encoded by the *pcbAB* gene [3-5]. The last steps include cyclization of the ACV tripeptide to isopenicillin N (catalyzed by the isopenicillin N synthase encoded by the *pcbC* gene) and substitution of the L-\( \alpha\text{-aminoacidyl} \) side chain of isopenicillin N by aromatic acyl side chains (performed by the isopenicillin N acyltransferase encoded by the *penDE* gene) [6, 7]. Previous activation of the aromatic acid by a specific aryl-CoA ligase [8, 9] is required.

The genes involved in the biosynthesis of \( \beta\text{-lactam} \) antibiotics are arranged in clusters in bacteria as well as in filamentous fungi [1, 10, 11]. In *P. chrysogenum*, the three penicillin biosynthetic genes are clustered together in a DNA region, which is amplified in tandem repeats in high producing strains, together with other ORFs [12, 13, 14].

Besides penicillin, *P. chrysogenum* and other filamentous fungi synthesize different secondary metabolites, such as roquefortine and the characteristic green pigment [15]. Multidomain enzymes such as polyketide synthases (PKSs) and nonribosomal peptide synthetases (NRPSs) are in charge of the biosynthesis of secondary metabolites [16-19]. A common feature to these enzymes is that they require a post-translational modification to become active. The apoprotein form (inactive) becomes active (holo form) after the covalent attachment of the \( 4'\text{-phosphopantetheine moiety} \), which derives from coenzyme A [20]. Phosphopantetheine has been found to be present in ACV synthetase and in other peptide synthetases [21]. The binding site of this moiety is a serine, which is conserved in all acyl carrier and peptidyl carrier domains present in fatty acid synthases PKSs and NRPSs, respectively [22]. Phosphopantetheine is added to the apoprotein by an enzyme known as a \( 4'\text{-phosphopantetheinyl transferase (PPTase)} \) [20, 23]. In the case of the ACVS, the acyl carrier domain (thiolation) of each of the three modules bears a conserved serine residue that binds a thiol-containing phosphopantetheine co-factor [21, 24]. This co-factor may in turn, form the thioester with the activated amino acid, recognized by the cognate activation (A) domain of each module.

Activation through phosphopantetheine addition is not only found in enzymes of secondary metabolism. Fungi have a unique pathway for the biosynthesis of lysine via the \( \alpha\text{-aminoacidic acid} \). The \( \alpha\text{-aminoacidate reductase catalyses the conversion of} \ \alpha\text{-aminoacidate into} \ \alpha\text{-aminoacidate semialdehyde} [25-27]. This enzyme is synthesized as a catalytically inactive form, which is activated after the addition of a phosphopantetheinyl group by the PPTase [26, 28, 29].

PPTases have been classified into three major groups according to their substrate specificity. The first class includes the PPTases of bacterial acyl carrier protein synthases (ACPS-type), which are involved in the fatty acid synthesis [20] and in the activation of bacterial polyketide synthases [30]. The second type includes eukaryotic PPTases that constitute an integral domain of fungal type I fatty acid synthases (FASs) [31]. The third group, the Sfp-type PPTases, are twice the size of the ACPS-type enzymes and show a broader range of specificity. PPTases from this family are often associated with a biosynthetic pathway and are required for secondary metabolism in bacteria [20, 32] and for lysine biosynthesis in fungi [33].
Some strains of *P. chrysogenum* contain an amplification of the penicillin gene cluster (pen) region [12-14] and produce very large amounts of penicillin, thus requiring a high PPTase activity. This might be limiting for ACVS modification unless there is a gene duplication. Therefore, it was important to identify the gene encoding the PPTase of *P. chrysogenum* to elucidate the role of this protein in primary and secondary metabolism. In this work, we report the isolation and characterization of the gene encoding a stand-alone large (class III) PPTase of *P. chrysogenum* as well as the generation of overexpressing and lack-of-function mutants. Our results indicate that a single PPTase is involved in a wide spectrum of processes, including penicillin production, pigmentation and lysine biosynthesis, but not in fatty acid or roquefortine formation.

**EXPERIMENTAL**

**Strains and culture conditions**

*P. chrysogenum* NRRL-1951 (wild type) and Wis54-1255 strains, which contain a single copy of the penicillin gene cluster [12], were used as the parental strains in this work. The *P. chrysogenum* Wis54-1255 pyrG strain (an uridine auxotroph derived from the Wis54-1255 strain) and the high penicillin producing strains AS-P-78 and AS-P-99 were used to study the gene copy number. The lysine auxotroph L2/lys1 strain, a derivative of the L2 mutant strain [34] disrupted in the *lys1* gene (F. Teves et al., unpublished data) was used as control strain of lysine auxotrophy, respectively. Uridine auxotrophs were grown in presence of 140 µg/ml of uridine. Selection of transformants with plasmids carrying the ble maker was made using 30 µg/ml phleomycin. Lysine auxotrophs were grown in the presence of 255 µg/ml of lysine (1.75 mM). Fungal spores were plated on complex Power medium [35] or Czapek minimal medium (30 g/l sucrose, 2g/l NaNO₃, 0.5 g/l K₃HPO₄, 0.5 g/l MgSO₄.7H₂O, 0.01 g/l FeSO₄) and grown for seven days at 28ºC. In some experiments, 2.5% (v/v) olive oil and 2.5% (v/v) corn oil were added to the Czapek minimal medium.

*P. chrysogenum* liquid cultures were initiated by inoculating fresh spores in complex medium CIM (20 g/l corn steep solids, 10 g/l yeast extract, 58 mM sucrose, 50 mM calcium carbonate, pH 5.7) or defined DP medium [35] without phenylacetate. After incubation at 25ºC for 20 h in an orbital shaker (250 rpm), aliquots were inoculated in complex penicillim production medium CP (4 g/l potassium phenylacetate, 20 g/l pharmamedia, 50 g/l lactose, 0.03 M ammonium sulphate, 0.05 M calcium carbonate, pH 6.6) or defined DP medium with phenylacetate.

Competent cells of *Escherichia coli* DH5α strain were used for amplification and isolation of plasmid DNA. They were grown in Luria- Bertani Broth medium (LB) with ampicillin (100 µg/ml).

**Transformation of *P. chrysogenum* protoplasts**

*P. chrysogenum* protoplasts were obtained and transformed as previously described [36,37]. Selection of transformant clones was achieved by complementation of the uridine auxotrophy or by resistance to phleomycin (final concentration of 30 µg/ml).

**Southern and Northern blotting**

DNA and RNA isolation, Southern and Northern blotting were carried out as described before [8,13,38]. For hybridizations different probes were used; i) a 1284-bp *ppt* gene, ii) a 366-bp fragment located inside the *ppt* gene (see below), iii) a 524-bp *EcoRV* fragment, which includes the initial 96 bp from the *ppt* gene plus 428 bp of the 5’ region, iv) a 429-bp fragment located inside the third exon of the *penDE* gene (from...
nucleotide 727 to 1155), v) a fragment including the last 1209-bp of the pcbAB gene and vi) β-actin gene. The signal provided by the Northern blotting was quantified by densitometry using the “Gel-Pro Analyzer” software (Media Cybernetics). The transcript levels were determined comparing the intensity of the ppt mRNA signal to the β-actin mRNA signal (IOD/IOD β-actin).

Cloning of the ppt gene

In order to generate a DNA probe for the isolation of the P. chrysogenum ppt gene, PCR was carried out using genomic DNA as template. Two oligonucleotides were designed based upon two conserved amino acid sequences present in the NpgA/CfwA protein (PPTase) of A. fumigatus and A. nidulans. A sense oligonucleotide, encoding the conserved motif FNVSHQ, was used as forward primer (Fppt): 5’-ttcaacgtcagccaccaa-3’.

As reverse primer, an oligonucleotide encoding the conserved motif WALKEA, was used (Rppt): 5’-cgcccttttgagcgccca-3’. Thirty cycles of 1 min denaturation at 94°C, 1 min annealing at 55 °C and 1 min extension at 72°C, gave rise to the amplification of a 366-bp band. This band was used as probe to screen a P. chrysogenum NRRL-1951 genomic library [12] constructed in the vector λGEM-12 (Promega, Madison, WI, USA). One positive phage was isolated after three consecutive rounds of hybridisation. DNA was isolated from this clone and digested with different restriction enzymes, electrophoresed on 0.7% agarose gels, transferred by Southern blotting and probed with the 366-bp DNA fragment. Positive bands were sequenced and analysed using the blast algorithms available at http://www.ncbi.nlm.nih.gov/BLAST/.

Intron analysis

Identification of introns was performed by RT-PCR using the “OneStep RT-PCR Kit” (Qiagen, Hilden, Germany) following the manufacturer’s instructions. Total RNA was extracted from mycelia grown for 48 h in CP using the “RNeasy Mini Kit” columns (Qiagen), following the manufacturer’s instructions. RNA was treated with RQ1 RNase-free DNase (Promega Corporation) following the manufacturer’s instructions. Oligonucleotides ppt1; 5’-atggtagaccccagtgtgtc-3’ and ppt2; 5’-ttaaaagcttggtaaatccc-3’ were used for this purpose. They were designed to amplify the whole ppt gene. The presence of introns was confirmed by sequencing.

Plasmid constructions

Plasmid p43gdh-ppt was constructed to overexpress the ppt gene in P. chrysogenum as follows: The ppt gene was amplified using primers pptamplF; 5’-tcacaatgccatggtagaccccagtgtg-3’ and pptamplR; 5’-cagcaagaaggcctttaaaagcttggtaaatc-3’ and was cloned in the NcoI-StuI sites of pIBRC43 [39] between the A. awamori gdh gene promoter (a very efficient promoter in ascomycetes) and the Saccharomyces cerevisiae cycl transcriptional terminator. Plasmid pKS-3188ppt contains the 3188-bp DNA fragment released after SalI digestion of the positive phage including the ppt gene. This fragment contains the ppt gene and flanking regions and it was inserted in the SalI restriction site of pBluescript KS +. For some experiments the 3188-bp fragment was amplified by PCR using primers 3188F; 5’-gtcgaccgaagtggtttcggtt-3’ and 3188R; 5’-gtcgacgcgggattcgatgctc-3’.

Plasmid pBG, containing the P. chrysogenum pyrG gene, has been previously described [38]. Plasmid pJL43-RNAi-ppt is a derivative of the pJL43-RNAi (Ullán et al., unpublished data) and was made for the attenuation of the ppt gene. It was constructed as follows: a 1055-bp exon fragment of the ppt gene, spanning positions 145-1199, was amplified by PCR using primers atppt1; 5’-acgttggtcctcagttcatagttatgc-3’ and atppt2;
5′-aatatgcacctcctccatggggagcca-3′ and subcloned into the NeoI site of plasmid pJL43-RNAi.

**Penicillin bioassays and HPLC analysis**

The benzylpenicillin secreted to the culture medium by *P. chrysogenum* was quantified by HPLC and bioassay with *Micrococcus luteus* as test microorganism. Benzylpenicillin and isopenicillin N analyses by HPLC were carried out as previously described [38].

**Roquefortine extraction and HPLC quantification**

Roquefortine C was determined by HPLC. The mycelium from 72-hour cultures in complex penicillin production medium CP, was washed with NaCl 0.9% and extracted in Falcon™ tubes with 20 mL of dichloromethane. The extraction mixture was sonicated for 30 minutes and centrifuged at 4500 rpm for ten minutes at 4ºC. The dichloromethane extract was transferred to a clean tube and evaporated to dryness in a rotary Vacuum Concentrator (RotaVapor-210, Buchi, Switzerland). The residues were re-dissolved in 150 μL of methanol, centrifuged and stored at -20ºC. Chromatographic determinations were performed on a Waters HPLC-system (Waters Corporation, Milford, Massachusetts, USA) consisting of a 996 Photodiode Array Detector, a binary pump, and a Symmetry reversed-phase C18 column (150 mm x 3.9 mm) of particle size 5 μm (Waters Corporation) at room temperature. The mobile phase consisted of acetonitrile and water containing 0.04 % trifluoroacetic acid with a flow-rate of 0.7 mL/min. A linear gradient ranging from (70:30) to (20:80) water-acetonitrile was used for 25 minutes. After 5 minutes at (20:80) the eluent composition was changed to starting conditions. A standard of pure roquefortine C was kindly provided by A. Fernández (Biomar S.A.; León, Spain). A stock solution of roquefortine C was prepared in methanol and stored at -20ºC. Working standards were prepared just before use by serial dilutions of the stock solution in methanol.

**RESULTS AND DISCUSSION**

**Cloning of the ppt gene from a *P. chrysogenum* genomic library**

To clone the *ppt* gene of *P. chrysogenum*, two primers (Fppt and Rppt) corresponding to conserved internal sequences from PPTases of the phylogenetically related fungi *A. fumigatus* and *A. nidulans*, were designed. They encode the conserved motifs FNVSHQ and WALKEA, respectively, which are present in the NpgA/CfwA protein (PPTase) of *A. fumigatus* and *A. nidulans*. A 366-bp band was amplified, sequenced and compared to the NCBI sequences using the BLAST algorithms. Significant similarities of the PCR product with PPTases (NpgA/CfwA) of *A. fumigatus*, *Neosartorya fischeri*, *A. clavatus* or *A. nidulans* were found. Therefore, the 366-bp fragment was used as probe to isolate the complete *ppt* gene from a *P. chrysogenum* NRRL-1951 genomic library in the Lambda phage vector [12]. One positive phage was isolated after three consecutive rounds of hybridisation and its DNA was analysed by Southern blotting with a probe containing the 366-bp DNA fragment (see supplementary material S1). One of the positive bands (the 3188-bp band released after the *SalI* digestion), was subcloned into a *SalI*-digested pBluescript KS+, thus generating pKS-3188ppt. Sequence of the 3188-bp region was identical to the sequence 4 reported in the patent application *P. chrysogenum* WO 2005/040369 (Accession No. CS086376), and revealed the presence of a 1284-bp ORF in the center of this region. Upstream of it, a 424-bp ORF (incomplete and present in antisense orientation) showed a strong similarity with a glutamine amidotransferase subunit (PdxT) of *A. terreus* (e-value: 4e-49; Positives: 80%) and with a glutamine...
amidotransferase (GATase_I) involved in pyridoxine biosynthesis of *A. fumigatus* (e-value: 4e-49; Positives: 81%) or *N. fischeri* (e-value: 1e-48; Positives: 81%).

In order to detect the presence of introns within the 1284-bp ORF, RT-PCR assays were carried out using primers ppt1 and ppt2. One 48-bp intron was detected spanning nucleotides 28-75 (data not shown). Sequences of the 5´ and 3´ intron splice sites were the consensus “GTGAGT” and “CAG”, respectively. This ORF encoded a PTTase (see below) and the gene was named *ppt* gene (for phosphopantetheinyl transferase).

The predicted protein sequence encoded by the cDNA of the *ppt* gene, contained 412 amino acids with an estimated molecular mass of 45.8 kDa. This protein was 51% homologous (38% identity, e-value 3e-64) to the *NpgA/CfwA* protein (PTTase) of *A. fumigatus*, 51% homologous to the PPTase of *N. fischeri* (38% identity, e-value 3e-62), 50% homologous (38% identity, e-value: 4e-59) to the *npgA* protein (PTTase) of *A. nidulans*, 48% homologous (33% identity, e-value 5e-23) to the PPTase of *Gibberella zaeae*, 46% homologous (33% identity, e-value 1e-18) to the PPTase of *Magnaporthe grisea* and 41% homologous (28% identity, e-value 8e-16) to hypothetical protein related to the *npgA* protein of *Neurospora crassa*. The protein product encoded by the *ppt* gene of *P. chrysogenum* closely resembles the stand-alone large size (class III) PPTases from several filamentous fungi and it shared conserved regions such as “LASQLLK”, “FNVSHQA”, “VGIDV” and “RLFYSIWALKEAYLKMTGDGLLASWIKDLEF”, which are present in the PPTases from several filamentous fungi (Fig. 1) and in the PPTases of other organisms, such as *Bacillus subtilis*, *Homo sapiens*, *Arabidopsis thaliana* or *Saccharomyces cerevisiae* [40]. This class of fungal PPTases includes the PPTase encoded by the *A. nidulans npgA* gene, which has been shown to be necessary for penicillin [40], lysine [41] and siderophore biosynthesis [42].

The *ppt* gene is present as a single copy in the genome of wild type and high penicillin producing strains of *P. chrysogenum*

It is well known that many high penicillin-producing strains contain multiple copies of the *pen* gene cluster [12]. To determine the *ppt* gene copy number, genomic DNA from the *P. chrysogenum* NRRL-1951 strain was isolated, digested with different restriction enzymes and hybridised with a probe containing the *ppt* gene. As shown in figure 2 only one band was detected with most of enzymes. Only *Apa*I (~6000 bp and ~700 bp) and *EcoR*I (~1600 bp and ~1100 bp) gave rise to two hybridisation signals because they cut inside the *ppt* gene. These results indicate that the *ppt* gene is present as a single copy in the genome of the wild-type strain of *P. chrysogenum*. The same hybridisation pattern was obtained when the genomic DNA of the high penicillin-producing strains *P. chrysogenum* AS-P-78 and AS-P-99, was digested with the same restriction enzymes (data not shown). These results evidence that only one copy of this gene exists in the genome of the low and high penicillin producing strains, fact that is consistent with the finding that the *ppt* gene is not located inside the 56.8-kb amplified region containing the *pen* gene cluster [13, 14].

**Amplification of the *ppt* gene in *P. chrysogenum* Wis54-1255. Overexpression of the *ppt* gene leads to increased levels of penicillin**

Overexpression of the *ppt* gene was achieved using plasmid p43gdh-ppt. This plasmid was co-transformed together with plasmid pBG (which contains the *pyrG* gene as selectable marker) into the *P. chrysogenum* Wis54-1255 *PyrG-* strain. Transformants were selected by complementation of the uridine auxotrophy. Ectopic integration of the full Pgdh-ppt-Tcyc1 cassette in four transformants was confirmed by Southern blotting.
(see supplementary material S2). One of these transformants (T11) was randomly selected for further studies. Northern blottings were carried out with RNA extracted at 48 h and 72 h from cultures of the Wis54-1255 strain and transformant T11 to confirm the overexpression of the ppt gene. As observed in figure 3A, transformant T11 showed approximately 3-fold higher steady-state levels of the ppt transcript than the parental strain and therefore, it was named Wis54-PPT+ strain.

HPLC analyses of the penicillin produced in cultures in DP medium indicated that the specific benzylpenicillin production (per mg of dry weight) of the Wis54-PPT+ strain at 72 hours was up to 30% higher than that of Wis54-1255 at the same time grown under identical culture conditions (Fig. 3B). Isopenicillin N (an intermediate of the penicillin pathway) production was also determined by HPLC in the Wis54-PPT+ strain cultured in complex CP medium without phenylacetic acid (to optimize isopenicillin N production). Isopenicillin N levels in the transformant T11 were two-fold higher than in the Wis54-1255 strain (Fig. 3C). This result indicates that overexpression of the ppt gene leads to an increase in the initial steps of antibiotic production, i.e. ACV formation or cyclization of ACV to isopenicillin N. Since the 4'-phosphopantetheine moiety is present in the ACVS [21] and it is necessary for the activation of this multidomain enzyme, an increase of the ACVS activity as a consequence of the ppt gene overexpression (giving rise to increased levels of the tripeptide ACV and therefore, to higher levels of antibiotic production), is more likely.

Characterization of mutants defective in PPTase activity. The ppt gene is essential for lysine biosynthesis but not for fatty acids formation

Plasmid pJL43RNAi-ppt (see Experimental Procedures) was used to generate loss-of-function mutants in the PPTase. After transformation, selection of transformants was done through phleomycin resistance. Since the A. nidulans PPTase has been reported as essential for lysine biosynthesis [41], those phleomycin resistant transformants that showed reduced growth on minimal medium or lysine auxotrophy, were selected. Only one clone out of 100 was completely auxotroph for lysine and full integration of the PgdA-ppt-reversePpcbC cassette was confirmed by Southern blotting (see supplementary material S3). This transformant showed a reorganization of the ppt locus. This fact was confirmed by Southern blotting (Fig. 4A), which was carried out using genomic DNA digested with SalI. A 524-bp EcoRV fragment (absent in the PgdA-ppt-reversePpcbC attenuation cassette, thus giving information only about the internal ppt gene) was used as probe. Results evidenced that the 3188-bp SalI fragment, which includes the endogenous ppt gene, had been reorganized as a 2100-bp band. This generates the deletion of the last ~191 bp of the ppt gene giving rise to a disrupted mutant, which was named Wis54-PPT−. Lysine auxotrophy of the ppt disrupted mutant was confirmed growing the Wis54-1255 strain, the Wis54-PPT− strain, and the lysine auxotroph L2/lys1− strain (as control) in Czapek minimal medium supplemented with 1.75 mM lysine (Fig. 4B). As observed in this figure, addition of lysine is completely essential for growth of the Wis54-PPT− strain. This indicates that the ppt gene encodes the PPTase in charge of activation of the α-aminoadipate reductase, which catalyses the conversion of α-aminoadipic acid into α-aminoadipate semialdehyde in the fungal lysine biosynthetic pathway [27]. It was also observed that growth was slower in the Wis54-PPT− strain than in the rest of strains. Since the A. nidulans PPTase, encoded by the npgA gene, is essential for siderophore biosynthesis [42], it is likely that the P. chrysogenum PPTase is involved in the maintenance of the intracellular pools of iron through siderophore biosynthesis. However, the lack-of-function mutant Wis54-PPT− was able to grow in lysine-
supplemented minimal medium without addition of siderophores. This indicates that although siderophores have been described in *P. chrysogenum* [43,44], those might not be essential, unlike the NRPS-mediated siderophore biosynthesis in *A. nidulans*, which has been shown to be completely necessary for growth [45].

A limitation in fatty acid biosynthesis could be another explanation to the growth defects observed in the Wis54-PPT− strain. To test this, the parental Wis54-1255 strain, the Wis54-PPT− mutant, and the L2/lys1− strain were grown on Czapek minimal medium supplemented with 2.5% (v/v) olive oil plus 2.5% (v/v) corn oil. Addition of oils to the minimal medium did not restore the growth rate of the Wis54-PPT− strain (see supplementary material S4). This indicates that there must be other PPTases different from that encoded by the *ppt* gene in charge of the activation of FAS in *P. chrysogenum*. In *S. cerevisiae* the subunit α of FAS is capable of self-pantetheinylation [31]. Probably the same mechanism occurs in *P. chrysogenum*, where a class of PPTase controls lysine biosynthesis and other class, present as an integral domain of fungal type I FASs, controls fatty acids biosynthesis. This may explain the lack of requirement of fatty acids in the Wis54-PPT− mutant.

The *ppt* gene is involved in penicillin and green pigment formation, but it is not required for roquefortine C biosynthesis

The Wis54-PPT− mutant took 10 days to develop conidiophores and spores in Power medium, unlike the Wis54-1255 strain, which sporulated in about 5 days. White cotton-like conidiophores, unlike the characteristic green conidiophores of the Wis54-1255 strain, were formed in this mutant (see supplementary material S5). The conidia remained colourless due to the inability to synthesize the green pigment, apparently a polyketide-derived compound. It has been reported that the *A. nidulans* wA gene encodes all or part of a PKS involved in the formation of a green pigment present in conidia [46]. The lack of green pigment formation in the *ppt* knockout mutant indicates that the PPTase encoded by the *ppt* gene is also involved in PKSs activation, specially in the activation of the PKS wA-encoded ortholog of *P. chrysogenum*.

The role of the *ppt* gene on secondary metabolism was evident when the penicillin production was tested using sporulated agar plugs (Fig. 5). As shown in this figure, penicillin biosynthesis was completely abolished in the Wis54-PPT− strain. The same results were obtained using filtrates from submerged cultures in complex CP medium (see supplementary material S5). The lack of antibiotic production was not due to a reorganization of the penicillin biosynthetic cluster in the Wis54-PPT− strain, as we confirmed by Southern blotting (see supplementary material S6). Instead, it is likely accounted by the absence of activity of the ACVS, since this NRPS requires phosphopantetheine-mediated activation of the constituent peptidyl carrier domain to catalyse the biosynthesis of the tripeptide ACV [5,21,24,47]. In addition, the isopenicillin N synthase, which is a nonheme Fe²⁺-dependent oxygenase [48] may have a reduced activity in the Wis54-PPT− mutant, since the availability of iron might become limiting because of a defect in the siderophore biosynthesis.

In addition to β-lactam antibiotics, *P. chrysogenum* is able to produce other secondary metabolites, such as the alkaloid roquefortine C [15]. The biosynthesis of this mycotoxin is poorly known; it is believed to be formed by a NRPS (the gene encoding this NRPS has not been cloned yet) that uses dimethylallyltryptophan as one of the precursors [49]. When the roquefortine C biosynthesis was analysed by HPLC in the Wis54-PPT− mutant and in the Wis54-1255 strain, no differences were found between these two strains (see supplementary material S7), which suggests that the biosynthesis of roquefortine may not require post-translational modification by the PPTase.
Alternatively, the presence of a second PPTase involved in the activation of the NRPS in charge of the biosynthesis of this alkaloid, cannot be excluded.

**Complementation of the Wis54-PPT strain with the ppt gene restores lysine biosynthesis, normal growth rate, pigmentation and penicillin production**

In order to confirm the role of the *P. chrysogenum* PPTase in lysine biosynthesis and secondary metabolism, the Wis54-PPT strain was complemented with plasmid pKS-3188ppt (see Experimental Procedures). Integration of the 3188-bp region, which includes the *ppt* gene, was confirmed by PCR (Fig. 6A). Transformants were selected by their ability to grow in presence of phleomycin (a characteristic of the Wis54-PPT strain) and lysine prototrophy restoration was confirmed in one of the transformants (randomly selected) (Fig. 6B). The transformant showed a growth rate similar to that of the Wis54-1255 strain. In addition, the green pigmentation of conidiophores and penicillin production were completely restored in the complemented transformant (see supplementary material S8).

All these data, taken together, indicate that the *P. chrysogenum* stand-alone large size (class III) PPTase encoded by the *ppt* gene is in charge of the post-translational activation of the α-aminoadipate reductase, ACVS and the NRPS involved in the green pigment formation, evidencing that this protein is involved in the post-translational control of both primary and secondary metabolism.

**ACKNOWLEDGEMENTS**

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REFERENCES


FIGURE LEGENDS

Figure 1. Alignment of the conserved motifs present in the P. chrysogenum PPTase to different PPTases from several filamentous fungi.

npgA (PPTase) from A. nidulans (Accession No. AAF12814) (npgAAnidu); PPTase NpgA/CfwA from Aspergillus fumigatus (Accession No. XP_755193) (npgAAsfm); protein NpgA related to null pigmentation protein from Neurospora crassa (Accession No. CAE76613) (npgANcras); hypothetical protein MGCH7_ch7g840 from Magnaporthe grisea (Accession No. XP_001522742) (PPTaseMgri) and hypothetical protein FG08779.1 from Gibberella zeae (Accession No. XP_388955) (PPTaseGzea). These proteins were aligned to the P. chrysogenum PPTase (PPTasePchr) using the ClustalX program. Note the strong conservation of amino acids 224-254.

Figure 2. Southern blot analysis of the ppt gene copy number in P. chrysogenum.

DNA from the P. chrysogenum NRRL-1951 wild type strain was digested with several restriction enzymes (A: ApaI; B: BamHI; E: EcoRI; H: HindIII; K: KpnI; N: NcoI; P: PstI; S: SacI; Sa: SalI; X: XhoI). The P. chrysogenum ppt gene was used as probe. The λ-HindIII molecular mass marker is indicated as M.

Figure 3. Amplification of the ppt gene in P. chrysogenum.

A) Northern blottings performed with samples taken after 48 h and 72 h from cultures of the transformant T11 (PPT+) and the Wis54-1255 strain (Wis54) grown in CP medium. The filter was hybridised to a probe containing a fragment of the ppt gene. As a control of RNA levels loaded in the gel, a probe with the β-actin gene was used. The steady-state levels of the ppta transcript of transformant T11 (PPT+) and Wis54-1255 strain were determined by densitometry. Transformant T11 was referred hereafter as Wis54-PPT+ strain. B) Representative HPLC chromatogram of the filtrates obtained from one of the liquid cultures (72 hours) of the Wis54-PPT+ and Wis54-1255 strains under penicillin production conditions. Three different experiments with duplicate flasks were done. Potassium benzylpenicillin (PenG) was used as control. C) Isopenicillin N specific production of the Wis54-PPT+ and Wis54-1255 strains after 24 h, 48 h and 72h of growth in CP medium without the addition of phenylacetic acid. Three different experiments with duplicate flasks were done and isopenicillin N levels were determined by HPLC. Three determinations were done for each value. Vertical bars indicate the standard deviation.

Figure 4. Disruption of the ppt gene leads to lysine auxotrophy.

A) Southern blotting performed with total DNA digested with SalI and probed with a 524-bp EcoRV fragment (grey box), which includes the first 96 bp of the ppt gene plus 428 bp of the 5′ region. Note the size reduction of the 3188-bp band containing the ppt gene in the Wis54-PPT− strain. The λ-HindIII molecular mass marker is indicated as M. B) Growth of Wis54-1255, the lysine auxotroph L2/lys1− and Wis54-PPT− strains in Czapek minimal medium with or without addition of 1.75 mM lysine.

Figure 5. Role of the ppt gene in penicillin production.

Bioassay of sporulated agar plugs showing that benzylpenicillin biosynthesis is completely abolished in the Wis54-PPT− strain grown on solid medium. The Wis54-1255 strain was used as control.

Figure 6. Complementation of the Wis54-PPT− strain with the ppt gene.
A) Electrophoresis of the PCR product obtained after amplification of the 3188-bp region, which includes the *ppt* gene. The PCR was carried out using DNA extracted from the complemented (Com), the Wis54-PPT⁻ (PPT⁻) and Wis54-1255 (Wis) strains and the primers specified in Experimental Procedures. Note the integrity of the 3188-bp band in the complemented and Wis54-1255 strains and the lack of amplification in the Wis54-PPT⁻ strain as a consequence of the *ppt* gene disruption. The authenticity of this band was confirmed by sequencing the PCR product. B) Growth of one randomly-selected transformant (Comp) obtained after complementation of the Wis54-PPT⁻ strain with the 3188-bp *ppt* region in Czapek minimal medium without lysine addition. The Wis54-1255, Wis54-PPT⁻ and L2/lys1⁻ strains were included as controls.
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Figure 1. García-Estrada et al.
Figure 2. García-Estrada et al.
Figure 3. García-Estrada et al.
Figure 4. García-Estrada et al.
Figure 6. García-Estrada et al.