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Biosynthetic Studies of Phomopsins Unveil Posttranslational Installation of Dehydroamino Acids by UstYa Family Proteins

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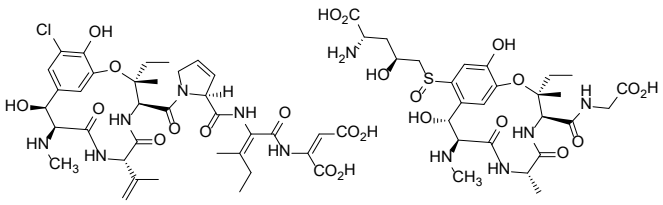
Abstract: UstYa family proteins (DUF3328) are widely and specifically distributed in fungi. They are known to be involved in the biosynthesis of ribosomally synthesized and posttranslationally modified peptides (RiPPs) and nonribosomal peptides, and possibly catalyze various reactions, including oxidative cyclization and chlorination. In this study, we focused on phomopsin A, a fungal RiPP consisting of unique nonproteinogenic amino acids. Gene knockout experiments demonstrated that three UstYa homologues, *phomYc*, *phomYd*, and *phomYe*, are essential for the desaturation of amino acid moieties, showing unprecedented function among UstYa family proteins. Sequence similarity network analysis indicated that their amino acid sequences are highly diverged, and that most remain uncharacterized, paving the way for genome mining of fungal metabolites with unique modifications.

Phomopsin A (**1**, Figure 1)[1] is a mycotoxin produced by the fungal pathogen *Phomopsis leptostromiformis* (*Diaporthe toxica*), which infects lupins and causes lupinosis disease in livestock.[2] It is also a potent antimitotic agent that binds to tubulin with high affinity.[3,4] Regarding its chemical structure and biosynthesis, **1** is a hexapeptide classified as a ribosomally synthesized and posttranslationally modified peptide (RiPP)[5,6] consisting of a 13-membered macrocyclic ether, chlorinated tyrosine (Tyr), and dehydroamino acids.[1] In 2016, Ding *et al.* reported a biosynthetic gene cluster of **1** and revealed that it is biosynthesized from the precursor protein PhomA.[7] Although the involvement of the tyrosinase-like gene *phomQ* was suggested by gene knockout experiments, details of the posttranslational modifications (PTMs) leading to the characteristic structure of **1** remain elusive. Among the unique structural features of **1**, the generation of dehydroamino acids, namely 3,4-dehydrovaline (dVal), 3,4-dehydroproline (dPro), 2,3-dehydroisoleucine (dIle), and 2,3-dehydroaspartic acid (dAsp), is of particular interest because it differs from the biosynthesis of dehydroalanine (Dha) and dehydrobutyrine (Dhb) residues that are found in bacterial RiPPs; Dha and Dhb are generated via phosphorylation or glutamylation of the hydroxy group of serine and threonine residues followed by elimination of phosphate or glutamate.[8,9] In contrast, the core sequence of the precursor protein PhomA and previous labelling experiments[10] suggest that the above dehydroamino acids are formed by oxidation of valine (Val), proline (Pro), isoleucine (Ile), and aspartic acid (Asp) residues, respectively. However, the enzymes responsible for this transformation have not yet been identified.



phomopsin A (**1**) ustiloxin B (**2**)

Figure 1. Chemical structures of phomopsin A (**1**) and ustiloxin B (**2**).

Prior to the identification of the phomopsin biosynthetic gene cluster, Umemura and co-workers identified the biosynthetic gene cluster for ustiloxin B (**2**, Figure 1), which contains a 13-membered macrocyclic ether similar to **1** (Figure 1).[11] Gene knockout experiments indicated that two functionally unknown genes, *ustYa* and *ustYb,* are involved in the biosynthesis; these genes include sequences that encode the DUF3328 domain, which is currently known as UstYa family proteins in Pfam (Pfam ID: PF11807; InterPro family: IPR021765).[12] Subsequently, heterologous expression in *Aspergillus oryzae* revealed that these two genes, in cooperation with the tyrosinase-like gene *ustQ*, are responsible for the oxidative cyclization and hydroxylation of the precursor protein UstA.[13] UstYa family proteins are widely distributed in the fungal kingdom,[14] and some members are involved in the biosynthesis of fungal RiPPs, such as asperipin-2a[14,15] and victorin.[16] Recently, we also determined that UstYa homologues are involved in the biosynthesis of the fungal nonribosomal peptide (NRP) cyclochlorotine. Three proteins, CctP2, CctO, and CctR, are responsible for the chlorination, transacylation, and hydroxylation of biosynthetic intermediates, respectively.[17] These studies suggested that UstYa homologues are involved in a variety of transformations, mostly oxidative ones, in the biosynthesis of fungal metabolites. In the biosynthetic gene cluster of **1**, five genes encoding UstYa homologues, *phomYa–Ye*, are present. Based on this observation, we hypothesized that UstYa homologues have a distinct function other than oxidative cyclization in the biosynthesis of **1**. In the present study, we conducted gene knockout experiments of the producing strain and identified that *phomYc*, *phomYd*, and *phomYe* are responsible for the desaturation of amino acid moieties. In addition, tyrosinase-like enzyme was found to be involved in the chlorination of the aromatic ring.

To gain insights into the biosynthesis of **1**, we independently conducted genome sequencing of the producing strain (ATCC 26115) because only the partial sequence was available in the public database. Although the candidate gene cluster was readily identified from the obtained data, several differences from the previous report were found (Additional Note in supporting information, page S3). First, the sequence of the precursor protein PhomA differed from the reported one. The reported sequence contains two different repeats, five repeats of YVIPID and three repeats of YVIPFD, that are converted to the final products. However, our sequence contains 10 YVIPID repeats (Figure S1). Second, the gene loci of *phomYb* and *phomA* were swapped. Third, another tyrosinase-like gene was newly identified between *phomQ* and *phomYd* (Figure 2A, Table S1). To avoid confusion, previously reported *phomQ* was called *phomQ1*, and the new gene found in this study was named *phomQ2*. The sequence identities of these gene products to UstQ are 39% and 52%, respectively. Fourth, while the similar nucleotide sequence to deposited *phomYa* was found between *phomF* and *phomP1*, it does not seem to be an ORF encoding a UstYa homologue. Therefore, we independently annotated *phomYa* gene in the neighboring region. We also identified small differences (e.g. single nucleotide mutations) in the nucleotide sequences of each ORF.

We then analyzed the metabolites of ATCC 26115 and identified **1**, the structure of which was confirmed by NMR and MS analyses (Figure 2B, S2–S4, Table S2). In addition, we identified minor metabolites **1a** and **1b**, with *m*/*z* 775 and 803, respectively (Figure S2). Based on the HRMS analyses, **1a** and **1b** were assumed to be *N*-desmethyl and *N*,*N*-dimethyl derivatives of **1**, respectively. This assumption was further supported by MS fragmentation analysis: both compounds yielded the same y series fragments as **1**, while the mass differences corresponding to the number of methyl groups were only observed in b series fragments that contained the N-terminal amino group (Figure S4).

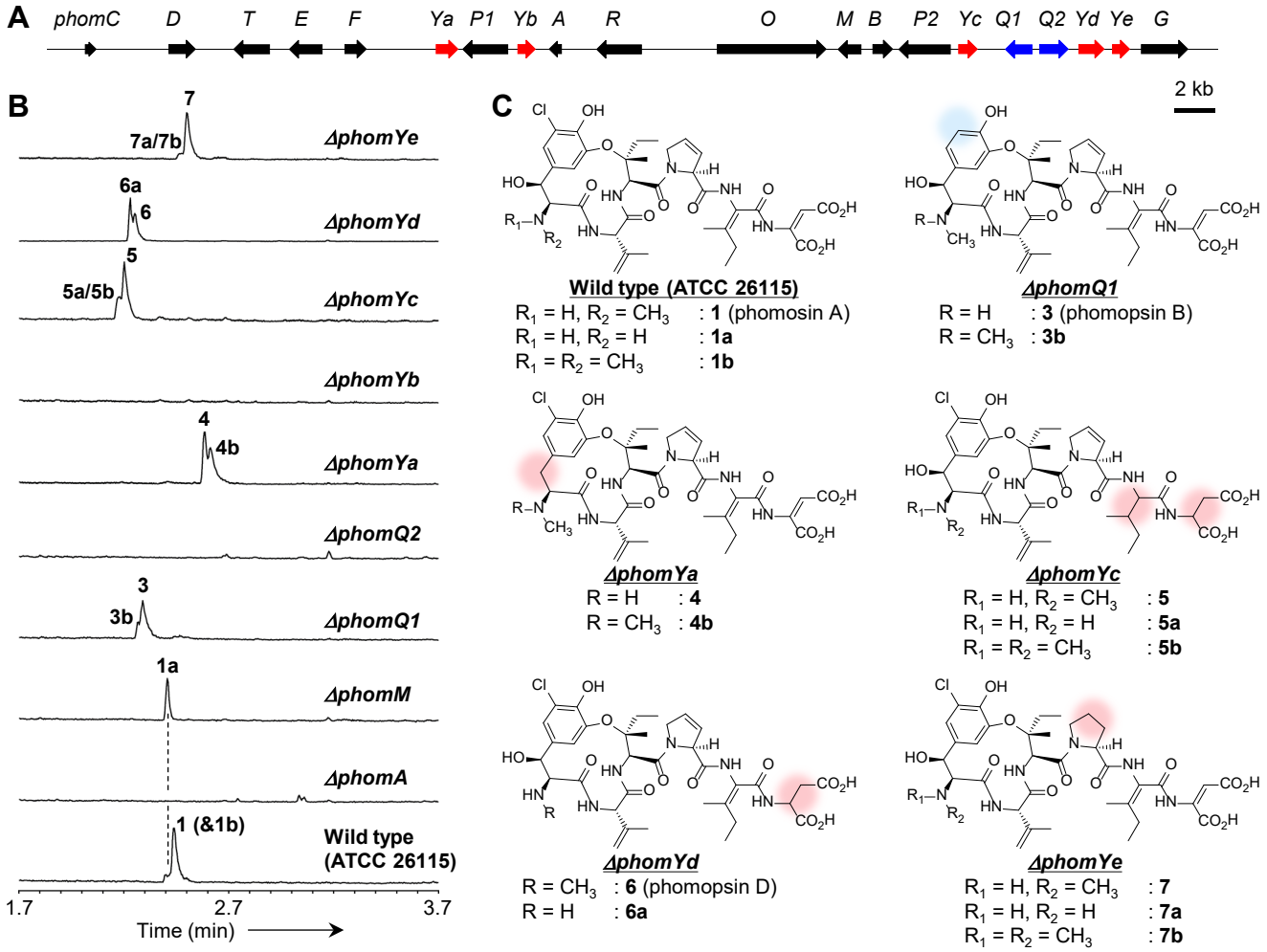


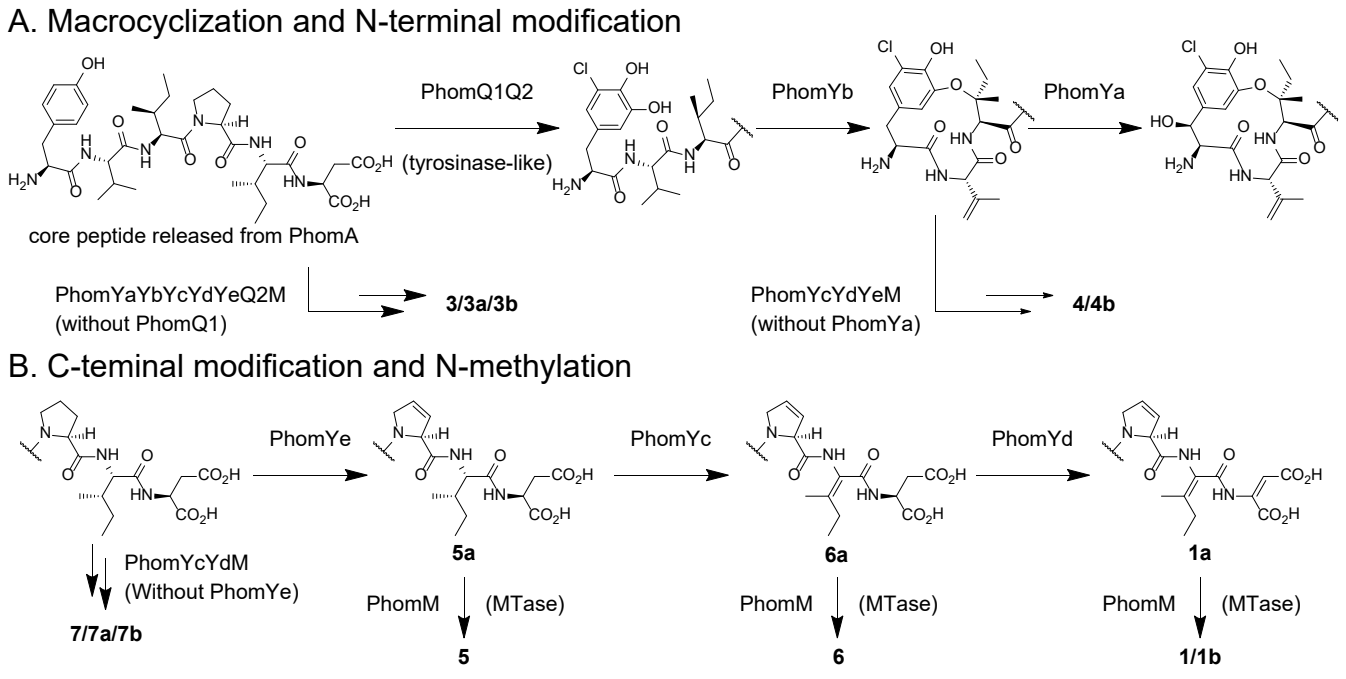
Figure 2. Analysis of the biosynthetic gene cluster of **1**. (A) Revised *phom* gene cluster. (B) LC-MS analysis of the metabolites extracted from each strain. Extracted ion chromatograms at *m/z* 741, 755, 759, 769, 773, 775, 777, 779, 787, 789, 791, 793, 803, 805, and 807 are shown to cover the possible des-, mono-, and dimethylated derivatives of phomopsin congeners produced by each mutant. The vertical scales are not identical. (C) Chemical structures of the compounds identified in this study. Stereochemistries are drawn based on those of **1**.

To functionally characterize the identified *phom* genes, we employed the *Agrobacterium tumefaicens*-mediated transformation (ATMT) method[18] to construct the deletion mutants of the producing strain. First, we examined the deletion

of the precursor gene *phomA* and the methyltransferase gene *phomM* to check the efficiency of this strategy (Figure S5AB, Table S2 & S3). As expected, deletion of *phomA* abolished the production of **1** and did not result in the accumulation of any related metabolites, thus establishing that PhomA is likely a biosynthetic precursor (Figure 2B). In contrast, deletion of *phomM* led to the accumulation of **1a** and did not produce *N*-methylated compounds **1** and **1b** (Figure 2B, S2). Previous *in vitro* experiments showed that PhomM catalyzes *N*-methylation of **1** to generate **1b**,[7] while its function in the biosynthesis remained obscure because **1** is a major product and **1b** is a minor product in the producing strain.[19] Our result and the above mentioned *in vitro* experiments unequivocally indicated that PhomM catalyzes two successive *N*-methylations, converting **1a** to **1b** via **1**.

After confirming that the ATMT method could be applied to ATCC 26115, we constructed deletion mutants of the two tyrosinase-like genes. Deletion of *phomQ1* abolished the production of **1** and caused the accumulation of **3** (Figure 2B, S5C). Based on the HRMS analysis, the molecular formula of **3** was determined to be $C_{36}H_{46}N_6O_{12}$, suggesting **3** to be phomopsin B, a dehalogenated derivative of **1**. MS fragmentation analysis further supported this conclusion: a b_2 ion consistent with the N-terminal structure of **3** was observed (Figure S4). This unexpected observation indicated that PhomQ1 functions as a halogenase in the biosynthesis. In contrast, *ΔphomQ2* did not produce **1** and other related metabolites (Figure 2B, S5D). This observation is similar to the result reported for deletion of orthologous *ustQ*, which is involved in the biosynthesis of **2**,[12] suggesting that these proteins have a similar function in the generation of the common 13-membered macrocycle. Considering the known hydroxylase activity of tyrosinase,[20–23] PhomQ2 and UstQ possibly catalyze the hydroxylation of Tyr.

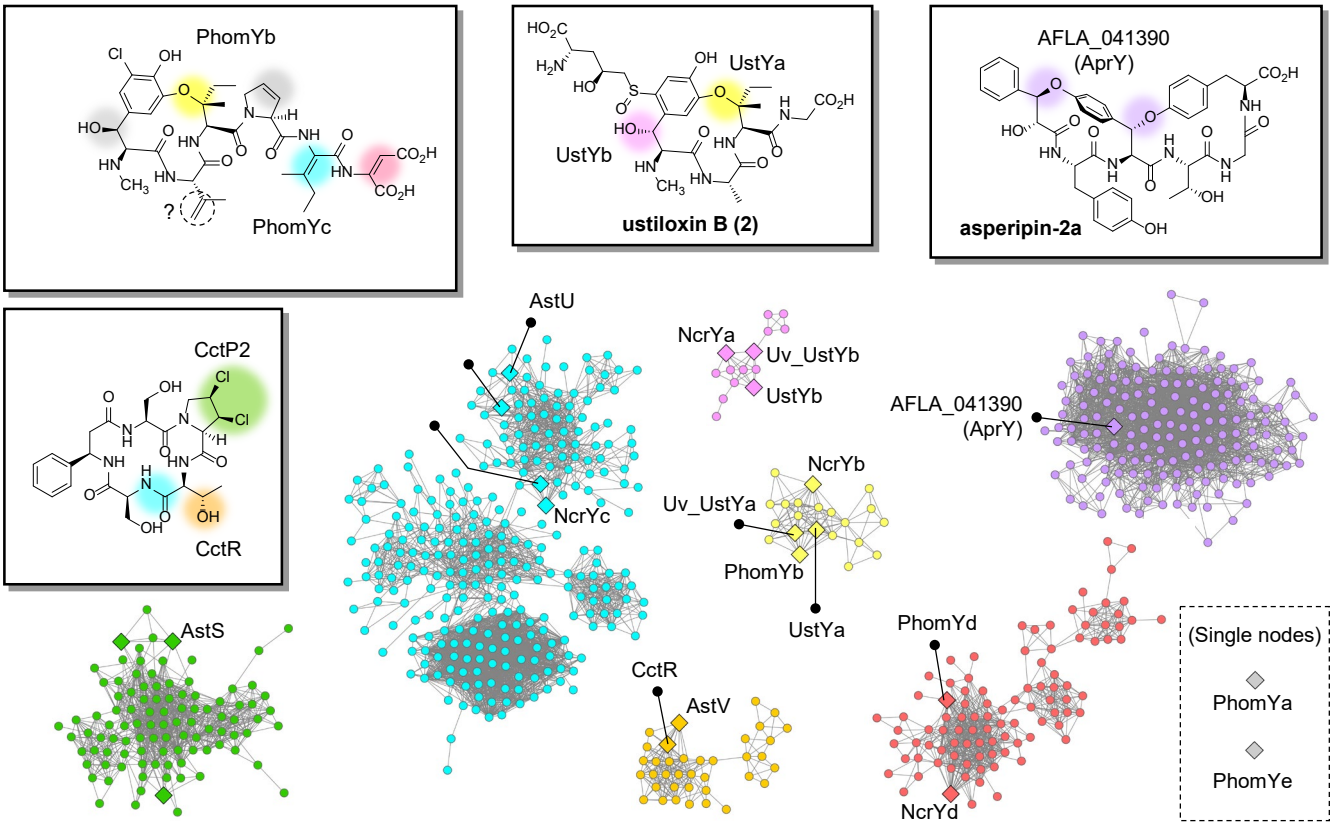
Finally, we turned our attention to the five *ustYa* homologues (Figure S5E–I). Deletion of *phomYa* resulted in the loss of **1** and accumulation of **4** (Figure 2B). The molecular formula of **4** was determined to be $C_{36}H_{46}N_6O_{11}Cl$, suggesting that **4** is a deoxy derivative of **1**. The proposed structure was further supported by MS fragmentation analysis (Figure S4). In the *ΔphomYb* strain, the production of **1** was abolished and related metabolites were not detected (Figure 2B). Production of **1** was also lost in strains *ΔphomYc*, *ΔphomYd*, and *ΔphomYe*, and they accumulated the distinct metabolites **5**, **6**, and **7**, respectively (Figure 2B). The molecular formulae of both **6** and **7** were determined to be $C_{36}H_{48}N_6O_{12}Cl$, while that of **5** was $C_{36}H_{50}N_6O_{12}Cl$, suggesting that one or two double bonds were lost in these compounds. MS fragmentation analyses readily identified that Asp was intact in the structure of **6** (Figure S4). In the case of **5**, both Ile and Asp were found to be unmodified (Figure S4), indicating that modification of the former is essential for dAsp formation. To further confirm the presence of unmodified amino acid residues in the structures of **5**, we conducted acid hydrolysis of these compounds, followed by treatment with dansyl chloride. As a result, we detected peaks for dansylated-Ile and dansylated-Asp by LC-MS analysis (Figure S6). In the case of **7**, the same b_3 fragment ion as in **1** was observed at *m*/*z* 434, while the b_5 ion was 2 Da larger than that of **1**. This observation indicates that either Pro or Ile remains intact. However, the lack of fragments derived from the cleavage of the amide bond between (d)Pro and (d)Ile hampered the delineation of the structure of **7** (Figure S4). Therefore, we conducted an NMR analysis with the purified compound, and it revealed that Pro was unmodified (Figure S3, Table S2). Interestingly, compounds **3**–**7** were accompanied by *N*-desmethyl (indicated by "**a**") and/or *N*,*N*-dimethyl ("**b**") derivatives of corresponding metabolites (Figure S2, S4) with varying ratios. This suggests that the PTMs introduced by each enzyme are important for substrate recognition by the methyltransferase PhomM.



Scheme 1. Proposed model for the biosynthesis of **1**. Note that the timing and order of each reaction could not be concluded from the present data.

Based on the above results, we propose the biosynthetic model for **1** shown in Scheme 1. To simplify the discussion, the proposed biosynthetic pathway is based on the assumption that the YVIPID hexapeptide is a starting material. The timing and order of proteolysis and PTMs are still unknown; the order of PTMs presented in Scheme 1 can be exchanged with each other unless otherwise noted. Two tyrosinase-like enzymes, PhomQ1 and PhomQ2, catalyze the chlorination and hydroxylation of Tyr, respectively. PhomYb, which shows sequence similarity to UstYa in ustiloxin biosynthesis (Scheme 2), is proposed to be involved in the construction of the macrocyclic structure that is common to **1** and **2**. The other four UstYa homologues do not show significant homology to UstYa and UstYb, and may be involved in PTMs that generate the unique structure of **1**. PhomYa is required for the hydroxylation of C_β of Tyr, which proceeds in the opposite stereochemistry to ustiloxin biosynthesis. PhomYc, PhomYd, and PhomYe are responsible for the biosynthesis of dIle, dAsp, and dPro, respectively. While dIle formation by PhomYc is indispensable for the installation of dAsp by PhomYd, the order of the other PTMs could not be elucidated by gene knockout experiments. As discussed later (*vide infra*), most of the biosynthetic enzymes likely have broad substrate specificity, and thus, there might be a metabolic grid from a precursor to **1**. We could not identify the gene(s) responsible for the biosynthesis of dVal. Currently, we hypothesize that PhomYb is a bifunctional enzyme that can catalyze oxidative transformations of structurally similar Val and Ile. Alternatively, other *phom* gene(s) or gene(s) outside the cluster might be necessary for the installation of dVal.

Notably, when one of the *phom* genes was knocked out, the remaining enzymes still converted altered intermediate(s) and produced phomopsin analogs lacking PTMs at specific position(s). The enzymes involved in fungal specialized metabolism often show relaxed substrate specificity, which contributes to the structural diversity of natural products. For example, in the biosynthesis of indole diterpene lolitrems, the prenyltransferase LtmE and the cytochrome P450 LtmJ accept most of the late-stage intermediates and construct the characteristic bicyclic structure of lolitremanes.[24] Another example is the halogenase PtmN in penitrem biosynthesis.[25] In such cases, only a few enzymes have substrate promiscuity, and deletion of one biosynthetic gene usually results in the accumulation of metabolite(s) that do not undergo downstream modifications.[26] In contrast, the phomopsin biosynthetic machinery is highly promiscuous and yields natural product-like analogs when one biosynthetic enzyme is omitted from the pathway. This unique feature would be advantageous in structure-activity relationship studies with the isolated compounds.



Scheme 2. Functional diversity of UstYa family proteins in the biosynthesis of fungal natural products. Selected clades are shown to visualize the relationship between PhomYs and other UstYa family proteins in the related pathway (see Figure S7A for full SSN).

The functional characterization of UstYa family proteins involved in the biosynthesis of **2**,[13] asperipin-2a,[15] and cyclochlorotine[17] has revealed their involvement in a variety of transformations (Scheme 2). This study found that UstYa family proteins are involved in the desaturation of amino acid moieties, adding new repertoires to the unique class of proteins. This

remarkable versatility is comparable to well-known oxidation enzymes such as cytochrome P450s[27] and non-heme iron enzymes[28] and represents the potential of UstYa family proteins as multifunctional oxidases specifically distributed in fungi. Sequence similarity network (SSN) analysis showed that PhomYb is grouped with UstYa involved in the biosynthesis of **2** (Scheme 2, Figure S7A, Table S5), suggesting their common role in the construction of the macrocyclic structure shared by **1** and **2**. Clustering of orthologous proteins involved in the biosynthesis of structurally related cyclochlorotines (CctO/P2/R) and astins (AstU/S/V)[29] was also observed. These observations indicate that SSN analysis can be used to predict the function of UstYa family proteins with close similarity. Interestingly, we found a *phom*-like gene cluster (*ncr* cluster) in the flanking region of the *poi-2* gene of *Neurospora crassa* OR74A (Figure S8). The *poi-2* gene is highly and specifically expressed in starved and sexual tissue, and its product is thought to be important for the mating response.[30] Considering that Poi-2 contains PhomA-like hexapeptide repeats flanked by the Kex-2 recognition sequence, we assumed that this protein serves as a precursor of the RiPP pathway that includes PTMs by four UstYa homologues, NcrYa–Yd, a tyrosinase-like protein NcrQ, and a methyltransferase NcrM (Figure S8). As NcrYa, NcrYb, NcrYc, and NcrYd are clustered with UstYb, UstYa/PhomYb, PhomYc, and PhomYd, respectively (Scheme 2), Poi-2 or its processed fragment likely undergoes hydroxylation, macrocyclization, and dehydrogenation to yield a phomopsin-like cyclic peptide. Poi-2 contains five different kinds of core peptide repeats, suggesting that the Ncr biosynthetic machinery is highly promiscuous and accepts these peptides as substrates (Figure S8).

While we can see the clustering of closely related homologues that are involved in the same type of modifications on similar substrates, proteins with similar functions do not fall into the same clade in most cases. Possible hydroxylases UstYb, PhomYa, and CctR are classified into distinct clades, and so too are putative dehydrogenases (PhomYc–Ye) and macrocyclases (AFLA_041390 and UstYa/PhomYb). Moreover, a putative dehydrogenase, PhomYc, is clustered with CctO, which was proposed to be a transacylase.[17] We also conducted multiple sequence alignment and recognized that only limited number of residues are conserved among selected UstYa homologues (Figure S7B). Two HXXHC motifs[14] were conserved in most proteins, while CctO and AstU were found to be exceptional cases. These observations indicate that the amino acid sequences of UstYa family proteins are highly diverged. Therefore, characterization of individual proteins is still necessary to elucidate the function of each protein. The biosynthesis of victorins remains an intriguing topic—they are elaborate RiPPs, and their biosynthetic process likely involves a multitude of UstYa homologues. Although they are expected to affect oxidative transformations and chlorinations,[16] we could not predict their function due to the lack of significant similarities to characterized proteins (Figure S7A).

In addition to the above examples, the possible involvement of UstYa family proteins in other fungal metabolic processes has been reported. A fungal polyketide-nonribosomal peptide hybrid atpenin is one example that likely involves a UstYa homologue in its biosynthesis.[31] As noted previously,[17] ApnIII is classified into the clade involving CctP2 and AstS and is likely involved in the chlorination. UstYa homologues may also be involved in the modification of fungal sesterterpene betaestacins (Figure S9)[32] and basidiomycota RiPP borosins.[33] Overall, UstYa family proteins are likely to be responsible for a variety of transformations required for the biosynthesis of fungal metabolites, irrespective of compound type. Given that the SSN shows that most family members remain uncharacterized, a genome mining approach will aid in the identification of unique fungal metabolites that undergo modifications mediated by UstYa family proteins.

In summary, we conducted genome sequencing of *P. leptostromiformis* ATCC 26115 and identified a phomopsin biosynthetic gene cluster that is slightly different from that previously reported. Gene knockout experiments unambiguously revealed that PhomQ1 (PhomQ) is involved in the chlorination of Tyr, demonstrating the unprecedented function of tyrosinase-like enzymes. Furthermore, UstYa family proteins were found to be responsible for the generation of dehydroamino acid moieties in addition to their known functions in oxidative cyclization and hydroxylation. To identify the gene(s) responsible for dVal formation, we are focusing on heterologous reconstitution of the pathway. As noted in the cyclochlorotine pathway,[17] expression of membrane transporters might be crucial for such experiments. Bioinformatic analysis indicated that the UstYa family comprises a diverse array of proteins whose function is largely unknown, creating good opportunities for genome mining of fungal metabolites using these proteins as probes. Although a series of genetic experiments collectively suggested that these proteins are biocatalysts involved in various transformations, their exact function remains elusive. To answer this question, we are currently working on the biochemical characterization of this enigmatic class of proteins.

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Keywords: Peptides • Biosynthesis • Natural products • Enzymes • Halogenation

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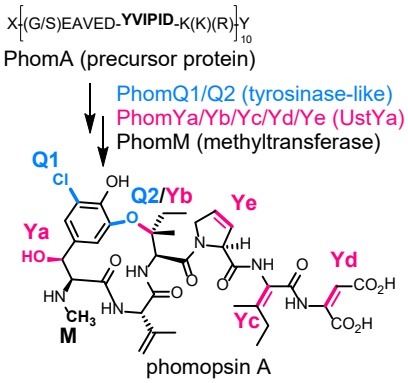

The biosynthesis of the fungal ribosomally synthesized and posttranslationally modified peptide phomopsin was elucidated by gene inactivation experiments. Notably, chlorination by tyrosinase-like enzyme PhomQ1 and desaturation of amino acid moiety by UstYa family (DUF3328) proteins PhomYc/Yd/Ye were uncovered. Besides the known function of UstYa homologues, this study unveiled the remarkable functional diversity of these fungi-specific proteins.