Biosynthesis of the mycotoxin tenuazonic acid by a fungal NRPS–PKS hybrid enzyme

Choong-Soo Yun1,*, Takayuki Motoyama1,* & Hiroyuki Osada1

Tenuazonic acid (TeA) is a well-known mycotoxin produced by various plant pathogenic fungi. However, its biosynthetic gene has been unknown to date. Here we identify the TeA biosynthetic gene from Magnaporthe oryzae by finding two TeA-inducing conditions of a low-producing strain. We demonstrate that TeA is synthesized from isoleucine and acetoacetyl-coenzyme A by TeA synthetase 1 (TAS1). TAS1 is a unique non-ribosomal peptide synthetase and polyketide synthase (NRPS–PKS) hybrid enzyme that begins with an NRPS module. In contrast to other NRPS/PKS hybrid enzymes, the PKS portion of TAS1 has only a ketosynthase (KS) domain and this domain is indispensable for TAS1 activity. Phylogenetic analysis classifies this KS domain as an independent clade close to type I PKS KS domain. We demonstrate that the TAS1 KS domain conducts the final cyclization step for TeA release. These results indicate that TAS1 is a unique type of NRPS–PKS hybrid enzyme.

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The mycotoxin tenuazonic acid (TeA), (5S)-3-acetyl-1, 5-dihydro-4-hydroxy-5-[(1S)-1-methylpropyl]-2H-pyrrol-2-one, is a tetracyclic acid derivative first isolated from the broth of an Alternaria tenius culture. Alternaria is a ubiquitous plant pathogenic fungus that causes both spoilage of various food crops and fruits in the field and post-harvest decay, and TeA has been detected in various Alternaria-contaminated crops, fruits and vegetables. In addition, the plant pathogenic fungi Phoma sorgina (a pathogen of sorghum) and Magnaporthe oryzae (a pathogen of rice) reportedly produce TeA. TeA is the most toxic of the Alternaria toxins. It inhibits protein biosynthesis on ribosomes by suppressing the release of new protein. It also reportedly shows biological properties including antitumor, antibacterial, antiviral and phytotoxic activities.

TeA is thought to be a hybrid of an isoleucine and two acetates. Due to its tetracyclic acid-containing structure, TeA is also expected to be a product of a polyketide synthase and non-ribosomal peptide synthetase (PKS–NRPS) hybrid enzyme. There are two types of fungal PKSs. One is iterative type I PKSs, consisting of multiple catalytic domains that contain non-ribosomal peptide synthetase (PKS–NRPS) hybrid enzyme.

Typical fungal secondary metabolite compounds polyketides and non-ribosomal peptides are synthesized by PKSs and NRPSs, respectively. There are two types of fungal PKSs. One is iterative type I PKSs, consisting of multiple catalytic domains that contain non-ribosomal peptide synthetase (PKS–NRPS) hybrid enzyme. There are two types of fungal PKSs. One is iterative type I PKSs, consisting of multiple catalytic domains that contain non-ribosomal peptide synthetase (PKS–NRPS) hybrid enzyme.

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Results
Identification of the TeA biosynthetic gene. Secondary metabolite production in microorganisms is regulated by environmental conditions for adaptation. For example, OSM1, a Hog1-related mitogen-activated protein kinase in M. oryzae, is reportedly involved in the response to environmental signals such as hyperosmotic stress. Therefore, we hypothesized that OSM1 is also involved in secondary metabolism regulation in M. oryzae. To confirm this hypothesis, we used M. oryzae to construct OSM1 (MGG_01822) knockout strains (Δosm1; Supplementary Methods and Supplementary Fig. 2) and analysed their metabolites with ultra performance liquid chromatography/mass spectroscopy (UPLC/MS). The production of one metabolite was induced in the Δosm1 strains under static culture conditions (Fig. 1b). We then obtained the ultraviolet pattern and mass spectra of the induced metabolite, which had a molecular mass of 197.2 (m/z = 196.2 [M-H]−) corresponding to the fungal secondary metabolite TeA (Fig. 1a, Supplementary Fig. 3). Although a previous report described the production of TeA in M. oryzae, the M. oryzae Kita 1 strain used in this study did not produce a detectable amount of TeA under our laboratory culture conditions (Methods section). In addition, while screening compounds that induce the production of secondary metabolites, we found that 1% dimethylsulphoxide (DMSO) also induced TeA production (14 mg l−1) in M. oryzae under static culture conditions (Fig. 1b). Although a number of studies have detected TeA in plants infected with TeA-producing fungi, the biosynthetic gene of this compound has been unknown to date. Thus, we undertook this study to identify the biosynthetic gene of TeA in M. oryzae. Two TeA-inducing culture conditions enabled us to explore the TeA biosynthetic gene in this organism.

Figure 1 | Tenuazonic acid biosynthetic gene identification. (a) Chemical structure of TeA. Red portions indicate the tetracyclic acid structure. (b) UPLC analysis of metabolites extracted from strains of M. oryzae Kita 1 (wild type (WT)), Δosm1 and DMSO-added culture conditions. Each strain was static-cultured for 5 days at 25°C. Arrows indicate TeA peak. (c) Signal intensities of OSM1, MGG_07803 and six PKS–NRPS genes in a microarray analysis with WT (black), Δosm1 (white) and strains cultivated under DMSO-added culture conditions (grey).
To identify the TeA biosynthetic gene, we performed a custom DNA microarray analysis using total RNA of *M. oryzae* extracted under the two TeA-inducing culture conditions and non-inducing normal culture conditions. TeA is expected to be the product of a PKS–NRPS hybrid enzyme, and the genome sequence of *M. oryzae* has six genes encoding such PKS–NRPS enzymes. However, the transcriptional levels of these genes were unchanged under TeA-inducing conditions. Of the remaining secondary metabolism-related genes, only one gene (*MGG_07803*) was found to be commonly upregulated (by more than twofold) under the two TeA-inducing conditions (Fig. 1c, Supplementary Table 1), suggesting that it is involved in TeA biosynthesis.

**Confirmation of the TeA biosynthetic gene.** To ensure that *MGG_07803* is the biosynthetic gene of TeA, we constructed an *MGG_07803* knockout. The knockout strain lost the capacity to produce TeA under the DMSO-added culture condition (Fig. 2a, Supplementary Fig. 4), confirming that this gene is the TeA biosynthetic enzyme. Accordingly, we renamed *MGG_07803* to *TAS1*. Because TAS1 is automatically annotated from the genome sequence of *M. oryzae*, we conducted rapid amplification of complementary DNA (cDNA) ends-polymerase chain reaction with the cDNA of *M. oryzae* that was cultured with 1% DMSO and identified an open reading frame (ORF) region (Supplementary Methods and Supplementary Fig. 5). Using this ORF, we constructed TAS1 overexpression strains by inserting the *A. oryzae* TEF1 promoter into the upstream of the TAS1 start codon via homologous recombination. UPLC/MS analysis of extracted metabolites from the resulting strain showed 28 mg l⁻¹ TeA production with the cDNA of *M. oryzae* MGG_07803 biosynthetic gene. Accordingly, we renamed *MGG_07803* to TAS1. Because TAS1 is automatically annotated from the genome sequence of *M. oryzae*, we conducted rapid amplification of complementary DNA (cDNA) ends-polymerase chain reaction with the cDNA of *M. oryzae* that was cultured with 1% DMSO and identified an open reading frame (ORF) region (Supplementary Methods and Supplementary Fig. 5). Using this ORF, we constructed TAS1 overexpression strains by inserting the *A. oryzae* TEF1 promoter into the upstream of the TAS1 start codon via homologous recombination. UPLC/MS analysis of extracted metabolites from the resulting strain showed 28 mg l⁻¹ TeA-producing capability without DMSO addition (Fig. 2a, Supplementary Fig. 4). This outcome also supported the conclusion that TAS1 is the TeA biosynthetic enzyme. TAS1 encodes an NRPS–PKS hybrid protein of 1,602 amino acids that consists of C, A and PCP domains in the NRPS portion and a KS domain in the PKS portion (Fig. 2b). The PKS portion of this domain structure is distinct from that of typical fungal PKS–NRPS hybrid enzymes with type I PKSs (Fig. 2b). Intriguingly, the NRPS portion of TAS1 also lacks the terminal release or cyclization domain (R or DKC), required for product release from the enzyme and generally present in fungal PKS–NRPS hybrid enzymes. In contrast, similar to type III PKSs, the PKS portion of TAS1 has only a KS domain. However, it shows very low similarity to known type III PKSs. Moreover, fungal PKS–NRPS hybrid enzymes have been reported but there are no reports of NRPS–PKS type fungal hybrid enzymes, even though bacterial NRPS–PKS enzymes have been identified. Therefore, TAS1 is a novel type of fungal secondary metabolite biosynthetic enzyme.

**Substrate specificity of TAS1.** On the basis of the structure of TeA, we expected that isoleucine and diketide are necessary for TeA biosynthesis. As TAS1 is a novel fungal NRPS–PKS enzyme with a PKS portion that has low similarity to that of type III PKSs, it is unknown whether this diketide is synthesized by TAS1. If TAS1 can synthesize this diketide, TeA can be synthesized by a single enzyme, TAS1, from isoleucine, acetyl-coenzyme A (CoA) and malonyl-CoA. Alternatively, TAS1 might use acetoacetyl-CoA as a source of diketide. To clarify this point, we prepared a recombinant enzyme and analysed its substrate specificity in vitro. The 173-kDa TAS1 protein was overexpressed in a protease-deficient yeast strain BJ5464 using *Saccharomyces cerevisiae* 2 μm episomal vector carrying the genes-encoding TAS1 under the control of the ADH1 promoter. To obtain the active form of TAS1, the 4’-phosphopantetheinyl transferase (PPTase; *MGG_17878*) of *M. oryzae*, which catalyses the essential post-translational activation of carrier proteins of NRPS and PKS, was coexpressed as a non-tagged protein. The purified C-terminally His-tagged recombinant TAS1 protein synthesized TeA as a single enzyme from isoleucine with acetoacetyl-CoA, not acetyl-CoA and malonyl-CoA (Fig. 3a,b), which clearly indicated that TAS1 is the TeA biosynthetic enzyme. The *k*<sub>cat</sub> was determined to be (14.9 ± 0.3) × 10⁻³ min⁻¹, and the *K*<sub>M(app)</sub> was (4.3 ± 3.1) × 10⁻³ mM for isoleucine. For acetoacetyl-CoA, the *k*<sub>cat</sub> was determined to be (13.1 ± 2.0) × 10⁻³ min⁻¹, and the *K*<sub>M(app)</sub> was (1.2 ± 0.1) × 10⁻³ mM. For ATP, the *k*<sub>cat</sub> was determined to be (15.3 ± 0.2) × 10⁻³ min⁻¹, and the *K*<sub>M(app)</sub> was (8.1 ± 0.5) × 10⁻³ mM (Supplementary Fig. 6).

**Homologue search and phylogenetic analysis.** To investigate the distribution of this type of hybrid protein, we conducted a Basic
Local Alignment Search Tool (BLAST) search for proteins with sequences similar to that of TAS1 via the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) and Alternaria genomes database (http://alternaria.vbi.vt.edu). Alternaria species are known to be major TeA producers. Homologue proteins that share 50% amino acid identity with TAS1 were found in Alternaria genomes database and their domain structures were identical to that of TAS1 (C–A–PCP–KS). TAS1 homologue proteins in Alternaria spp. were highly conserved and share over 97% identity. In addition, proteins with domain structures identical to that of TAS1 were also found in other fungal species from the National Center for Biotechnology Information database, which suggests that this type of hybrid enzyme is distributed in Ascomycota and Basidiomycota fungi (Supplementary Table 2).

However, similar protein sequences matched with TAS1 from bacterial sources had domain structures that differed considerably from that of TAS1. These proteins had a typical PKS structures consisted of KS, AT and ACP domains (Supplementary Table 2) at the end of the NRPS. This difference suggests that the C–A–PCP–KS domain structure of the NRPS–PKS hybrid enzyme is specific to fungi. We also conducted phylogenetic analysis of the KS domain in TAS1 with other KS domains from types I, II, III PKSs and PKS–NRPS hybrid proteins including the top five protein sequences from fungal and bacterial sources extracted from the TAS1 BLAST search result (Supplementary Table 2). Despite the single KS domain structure of the PKS portion, the KS domain of TAS1 was not classified as a type III PKS (Fig. 4, Supplementary Fig. 7). In addition, the KS domains from fungal PKS–NRPS hybrid enzymes were classified into one independent clade; however, the KS domain of TAS1 was not classified therein. Interestingly, protein sequences similar to TAS1 in bacteria were classified into an independent bacterial clade of type I PKSs with other proteins that reportedly interact with NRPS modules, including NRPS–PKS hybrid proteins. In contrast, the KS domain of TAS1 formed a new clade with the homologues from fungal sources, which was close to the type I PKSs (Fig. 4, Supplementary Fig. 7). This outcome indicates that TAS1 is a unique type of NRPS–PKS hybrid enzyme.

To further analyse TAS1 homologues, we conducted phylogenetic analysis of all KS domains, except for three homologues that have a truncated C-terminal KS domain (Supplementary Table 2), extracted from BLAST search results. All KS domains from TAS1 homologues were also classified into an independent clade different from type I PKS clades (Supplementary Fig. 8). In addition, TAS1 homologues were divided into four sub-clades. Sub-clade A has five KS domains and includes the TAS1 from M. oryzae and a TAS1 homologue from A. alternata. These two strains were already known to produce TeA. This suggests that strains have a TAS1 homologue in the sub-clade A are TeA producer. Interestingly, sub-clade B and C consists only of homologues from Ascomycota, whereas sub-clade D mainly contains homologues from fungi that infect insects, except for Fusarium spp.

**Functional analysis of the TAS1 KS domain.** Generally, the KS domain in the PKS enzyme is responsible for the control of polyketide product chain length via ketide extension. However, TAS1 uses acetoacetyl-CoA as a source of diketide to produce TeA, which means that ketide extension to yield the diketide (acetoacetyl-CoA) is unnecessary for TeA synthesis. Thus, we investigated whether the KS domain of TAS1 is necessary for TeA biosynthesis. We constructed the KS domain deleted TAS1 overexpression strains and found that the constructed strains lost the capability to produce TeA (Fig. 5a, Supplementary Fig. 9). This result showed that the KS domain is necessary for TeA biosynthesis. For further confirmation, we prepared separately expressed recombinant proteins with the C–A–PCP and KS domain portions of TAS1 by using a cell-free protein synthesis system (Supplementary Fig. 10a). Analysis of the TeA biosynthetic capability of these proteins showed that TeA was produced only when the C–A–PCP domains and the KS domain coexisted, which clearly indicated that the KS domain of TAS1 is indispensable for TeA biosynthesis (Fig. 5b). However, the exact role of the KS domain in TeA biosynthesis remains unknown.

From the perspective of the domain structure of TAS1, we proposed a model for TeA biosynthesis and hypothesized that the final cyclization step in TeA production is conducted by the KS domain (Fig. 5c). To support this hypothesis, we synthesized an artificial intermediate of TeA biosynthesis using N-acetyl-L-leucine–SNAC (SNAC), N-acetoacetyl-L-Ile-SNAC (Supplementary Methods and Supplementary Fig. 11), to analyse the cyclization capacity of the KS domain with the KS and C–A–PCP domain proteins synthesized with the cell-free system. The results showed that only the KS domain protein produced TeA, which indicated that this TAS1 domain is responsible for the final cyclization step in TeA production (Fig. 5d). In addition, we confirmed the condensation capacity of activated isoleucine and acetoacetyl-CoA by C–A–PCP or KS domain proteins using L-Ile-SNAC (Supplementary Fig. 11). The results showed that the condensed product (N-acetoacetyl-L-Ile-SNAC) is only produced by the C–A–PCP domain protein from L-Ile-SNAC and acetoacetyl-CoA, which indicated that the condensation reaction takes place via the C domain rather than the KS domain in TAS1 (Supplementary Fig. 12).

**Figure 4** | Phylogenetic analysis of the KS domain in TAS1. Phylogenetic analysis of the TAS1 KS domain and other KS domains. Domains were aligned with MUSCLE, and the tree was constructed with the neighbour-joining method. Scale bar, 0.1 substitutions per site. The TAS1 KS domain is indicated with a red line and purple line indicates TAS1 homologue of A. alternata. The KS domains of other TAS1 homologues are indicated with blue lines.

![Phylogenetic analysis of the KS domain in TAS1](image-url)
Figure 5 | KS domain analysis. (a) UPLC analysis of metabolites extracted from TAS1 overexpression (OE::TAS1) and KS domain-disrupted TAS1 overexpression (OE::TAS1ΔKS) strains. Arrows indicate TeA peak. A 100 μM TeA standard (TeA ST) was also analysed. (b) UPLC analysis of enzyme reaction products. C-A-PCP domain proteins, KS domain proteins or both of TAS1 were used as enzymes. A 100 μM TeA ST was also analysed. In vitro reaction conditions are described in the Methods section. Arrow indicates TeA peak. SNAC, N-acetoacetyl-L-ile-SNAC. A control reaction was conducted with cell-free-synthesized soluble protein without messenger RNA. In vitro reaction conditions are described in the Methods section. Arrow indicates TeA peak. SNAC, N-acetyl cysteamine.

Discussion

The recent sequencing of fungal genomes has shown that fungi are richer in secondary metabolism-related genes than once believed. However, the majority of these genes are silent or quiescent under laboratory culture conditions. During the past decade, successful approaches such as epigenetic regulation, promoter exchange and heterologous expression have been applied to activate these silent gene clusters in fungi. In this study, we activated the TeA biosynthetic gene in M. oryzae via disruption of OSM1, showing the possibility that disruption of an environmental signal response system can influence some of the poorly expressed or silent secondary metabolite genes in M. oryzae.

TeA was first reported in 1957 by Rosett et al., however, the TeA biosynthetic gene was not identified in the nearly 60 years that followed despite many reports of TeA detection. The chemical structure of TeA suggested that TeA is synthesized by a PKS and NRPS hybrid enzyme, and fungal PKS–NRPS hybrid enzyme was considered as biosynthetic enzyme of TeA, because reports of NRPS–PKS type fungal enzymes were not forthcoming. The TAS1 that we have identified is the first reported fungal NRPS–PKS enzyme, which may explain why the TeA biosynthetic gene has remained unidentified for so long. Our result also shows the limitations of the in silico prediction of polyketide and non-ribosomal peptide biosynthetic pathways from genomic sequence data.

The KS domain of TAS1 is quite different from other KS domains. Phylogenetic position of the KS domain of TAS1 is nearest to PKS type I. However, it does not belong to any clade of fungal NR-, PR- or HR-PKS type I and is clearly separated from the clades of PKS type I KS domains (Supplementary Fig. 7). In contrast to fungal KS domains of PKS type I, the KS domain of TAS1 is non-iterative. Moreover, the conserved catalytic triad residues of the KS domain in TAS1 is different from that of type I PKSs (Cys–His–His) and identical to that of type III PKSs (Cys–His–Asn; Supplementary Fig. 13). The catalytic triads of fungal homologues of TAS1 are also identical to that of TAS1. However, the residues surrounding the catalytic triad of TAS1 are more frequently conserved in the KS domains of type I PKSs than in those of type III PKSs (Supplementary Fig. 13). According to the above data, we suggest that the KS domain of TAS1 can be classified into a new group different from KS domains of type I, II, and III PKSs.

TAS1 consists of an NRPS and a unique type PKS KS domain involved in cyclization and product release. Cyclization and product release generally take place via the thioesterase (TE) or Claisen cyclase domains in fungal type I PKSs. In contrast, there is no fused TE domain in most HR-PKSs and product release and cyclization require an in trans interaction with separate enzymes. In addition, the cyclization step of macrocyclic peptides that produced by fungal NRPSs is mainly conducted by a terminal condensation-like domain. Interestingly, there are two product release mechanisms in fungal PKS–NRPS hybrid enzymes, which differ from the single PKS or NRPS. The first is the final R domain mediated release mechanism. The R domain is thought to catalyse the reductive release from the sequence similarities to short-chain dehydrogenase/reductase family proteins. The second mechanism
is DKC, catalysed by the terminal domain (D). Extensive investigations of tenellin, fusaridione and α-cyclopiazonic acid (fungal PKS–NRPS products) biosynthesis suggested that the formation of tetramic acid derivatives via DKC is the product release mechanism. In the proposed model for TeA biosynthesis (Fig. 5c), the final TeA release mechanism is expected to be DKC. However, TAS1 does not contain any of these cyclization and product release related domains. In contrast, type III PKSs have only a KS domain and the elongation/cyclization step for polyketide production is conducted with a single enzyme. In addition, biosynthesis analysis of the polyketide RK-682, which contains tetrionic acid (an analogue of tetramic acid), showed that a KS-like enzyme (RKD) responsible for assembly of the tetronic acid moiety by catalysing intermolecular C–C and C–O bond formation. In other polyketides-containing tetrionic acid—chlorothricin and quartromicin—biosynthetic pathways also contain these type of KS-like enzymes. However, tetrionic acid ring formation in TeA occurs via an intramolecular reaction catalysed by a KS domain (Dieckmann cyclization).

To study the peptide cyclization capability of TE domain in bacterial NRPSs, SNAC derivatives were extensively used as artificial intermediates of the enzyme reaction. SNAC mimics the enzyme-phosphopantetheine moiety that carries enzymatic intermediates in the cyclization enzyme reaction. However, non-enzymatic self-cyclization and non-reactiveness in SNAC derivatives have also been reported. We synthesized N-α-acetacycl-t-leu-1-SNAC as an intermediate of TeA biosynthesis to investigate the cyclization capability of the KS domain in TAS1; however, synthesized N-α-acetacycl-t-leu-SNAC showed self-cyclization, the rate of which increased with increasing pH. Thus, we conducted the enzyme reaction at pH 6.5 to minimize self-cyclization and demonstrated that the KS domain in TAS1 is responsible for the final step of cyclization in TeA production.

In fungal type I PKSs, the KS domain generally recognizes acetoy-CoA as a starter unit to produce polyketide. However, the TAS1 KS domain recognizes acetoycl-t-leu hybrid to initiate the cyclization reaction to produce TeA. Acetoacetyl-CoA is an intermediate of the mevalonate pathway and some type III PKS enzymes also recognize acetoycl-CoA as a starter unit to produce polyketides.

The Cys–His–Asn catalytic triad that is conserved in the type III PKSs and KS domain of TAS1 may be involved in cyclization and product release related domains. In contrast, these cyclization and product release related domains are not present in the cyclization and product release related domains. This primer set can amplify 10 kb of the TAS1 ORF, which was used as a wild-type strain. The vector sequence of pBI121 between the right and left borders was amplified with 2% agar for plate culture if needed.

**DNA manipulation.** DNA isolation and manipulation were performed with a standard method. Genomic DNA was isolated from M. oryzae with a DNeasy plant total DNA isolation kit (Qiagen, Valencia, CA, USA). DNA fragments were isolated from agarose with a gel extraction kit (Qiagen). Plasmids were purified using a QiAprep Spin Miniprep kit (Qiagen). PCR was carried out with a DNAEngine Peltier thermal cycler (Bio-Rad, Hercules, CA, USA). DNA amplification via PCR was conducted with KOD-Plus-neo DNA polymerase (Toyobo, Osaka, Japan). DNA fragment ligation and circularization for vector construction was conducted via infusion reaction using an In-Fusion HD cloning kit (Clontech, Palo Alto, CA, USA). DNA sequencing was carried out with a BigDye terminator ver3.1 kit (Applied Biosystems, Foster City, CA, USA). Sequencing products were run on an automated 3730 × 1 capillary DNA analyzer (Applied Biosystems).

**MGG_07803 gene disruption.** MGG_07803 was disrupted by exchanging a whole-genome ORF with the hygromycin B resistance gene expression unit via homologous recombination. MGG_07803 gene disruptants were constructed as follows. Two kb of the upstream region of MGG_07803 was amplified from the genomic DNA of M. oryzae via PCR with the primers AM07803_UP-F and AM07803_UP-R (fragment 1). The hygromycin B resistance gene expression unit was amplified via PCR from pCSN45 (ref. 60) with primers 5HPH and 3HPH (fragment 2). Two kb of the downstream region of MGG_07803 was amplified from the genomic DNA of M. oryzae via PCR with the primers AM07803_DOWN-F and AM07803_DOWN-R (fragment 3). The vector sequence of pBI121 (Clontech) between the right and left borders was amplified from pBI121 via PCR with primers pBI121-RB and pBI121-LB (fragment 4).

All fragments were gel purified and cloned by using the In-Fusion cloning technology to yield pBI-M07803-JHPH. The In-Fusion reaction mixtures were used to transform E. coli DH5α, and transformants were selected with Km (30 μg ml−1). After the DNA sequences were verified, plasmid DNA containing the correct insert was transformed into A. tumefaciens, and the transformants were used for A. tumefaciens-mediated transformation. MGG_07803 disruptants were selected via the kanamycin resistance gene expression unit. The strains and plasmids used in this study are listed in Supplementary Table 3. The strains and plasmids used in this study are listed in Supplementary Table 3.

**TAS1 gene overexpression.** TAS1 gene overexpression strains were constructed as follows. Two kb of the upstream region of TAS1 was amplified from the genomic DNA of M. oryzae via PCR with primers OETAS1_UP-F and OETAS1_UP-R (fragment 1). The blasticidin S resistance gene expression unit was amplified from pBI101-ΔNolr (ref. 58) via PCR with primers pDH5α and 3HPH (fragment 2). The TEF1 gene promoter region of A. oryzae was amplified from A. oryzae genomic DNA via PCR with primers AoTEF1-F and AoTEF1-R (fragment 3). The TAS1 ORF was amplified from the CDNA of M. oryzae that was cultured with 1% DMSO via PCR with primers TAS1-F and TAS1-R (fragment 4). Two kb of the downstream region of the TAS1 gene was amplified from the genomic DNA of M. oryzae via PCR with primers OETAS1_DOWN-F and OETAS1_DOWN-R (fragment 5). The vector sequence of pBI121 between the right and left borders was amplified from pBI121 via PCR with primers pBI121-RB and pBI121-LB (fragment 6).

All fragments were gel purified and then cloned with an In-Fusion HD Cloning kit (Clontech) to yield pBI-OE:TAS1. E. coli DH5α and A. tumefaciens transformation was conducted in the manner described for MGG_07803 gene disruption. The strains and plasmids used in this study are listed in Supplementary Table 3. The strains and plasmids used in this study are listed in Supplementary Table 3. The strains and plasmids used in this study are listed in Supplementary Table 3.
type strains and the 5.4-kb bacterial S resistance gene expression unit containing region from TAS1 overexpression strains (Supplementary Fig. 4).

KS domain disruption. KS domain-disrupted strains were constructed as follows. Two kb of the upstream region of TAS1 was amplified from the genomic DNA of M. oryzae via PCR with primers AKS_UP-F and AKS_UP-R (fragment 1). The hygroycin B resistance gene expression unit was amplified from pCNS45 (ref. 60) via PCR with primers 3PH1P and 3PH2P (fragment 2). The TEF1 promoter region and the C-AMP domains of TAS1 were amplified from pBI-EQ/TAS1 via PCR with primers TEF1_CAT-F and TEF1_CAT-R (fragment 3). Two kb of the downstream region of the TAS1 gene was amplified from the genomic DNA of M. oryzae via PCR with primers AKS_DOWN-F and AKS_DOWN-R (fragment 4). The vector sequence of pBP121 between the right and left borders was amplified from pBP121 via PCR with primers pBP121-1R and pBP121-2R (fragment 5).

All fragments were gel purified and cloned by using the In-Fusion cloning technology to yield pBI-TASI-KS:HPH. E. coli DH5α and A. tumefaciens transformation was conducted in the manner described for MGG,07803 gene disruption. The KS domain of TASI disruptants were selected via PCR with primers AKS_CHK-F and AKS_CHK-R that hybridizes upstream of the KS domain and outside of the downstream region used for vector construction for TASI. This primer set can amplify 4.1 kb from wild-type strains and 2.9 kb from the KS domain disruptants (Supplementary Fig. 9).

Microarray analysis. An RNeasy Plant mini kit (Qiagen) was used to extract total RNA from static-cultured M. oryzae (wild type, Δosm1 and 1% DSMO-added condition) on day 5 of culture. Double-stranded cDNA synthesis (from 10 μg total RNA) was performed using a NimbleGen (Madison, WI, USA) according to standard protocols. A custom-designed microarray platform comprising single 60-mer probes designed against M. oryzae was used. Five non-overlapping 60-mer probes were designed against various parts of the single target gene, and 64,125 probes were finally designated against the total number of annotated genes. An average transcriptional intensity of five non-overlapping probes in the single gene was used as the transcriptional level of the gene.

Metabolite extraction and analysis. M. oryzae culture broth (100 μl) was extracted with four volumes of ethanol, evaporated with an N2 stream and dissolved in 200 μl methanol to analyse metabolites. UPLC/MS analysis was performed using an ACQUITY UPLC H-Class system (Waters Alliance, Milford, MA, USA) equipped with a mass spectrometer (API 3200, Applied Biosystems). The UPLC conditions were as follows: column, X TerraMSCI (Waters), 5 μm (2.1 × 150 mm); flow rate, 0.6 ml min⁻¹; solvent A, water-containing 0.05% formic acid; solvent B, acetonitrile. After the extracted sample was injected into a column equilibrated with 5% solvent B, the column was developed with a linear gradient from 5 to 100% solvent B over the course of 3.5 min and kept at 100% solvent B for another 2 min. Mass spectra were collected in the electrospray ionization-positive and electrospray ionization-negative modes.

Protein expression and purification from E. coli. PPTase (MGG,17878 of M. oryzae was overexpressed with the E. coli BL21(DE3)PlyS system (Takara). The PPTase ORF (978 bp) of M. oryzae was amplified from the genomic DNA via PCR with primers pET-MoPPT-F and pET-MoPPT-R. The pET19b(+)-vector was amplified from plasmid pETDuet6388 with primers pET_F and pET_R, and then two fragments were infused to obtain pET-MoPPT for the expression of N-terminally 6×His-tagged PPTase. The constructed pET-MoPPT sequence was confirmed after transformation into E. coli BL21(DE3)PlyS. After overnight culture at 37°C in LB with Km (50 μg ml⁻¹), 2 ml pre-cultured transformed cells were inoculated into 100 ml fresh LB with Km (50 μg ml⁻¹) and cultured at 37°C for 2 h. Isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM. After growth for 5 h at 30°C, the cells were harvested via centrifugation (5,000g) for 15 min at 4°C. The collected cells were suspended in 10 ml protein extraction buffer and sonicated five times on ice for 30 s each with 30-s intervals. Total cellular debris was removed by centrifugation (8,000g for 30 min). The supernatant was purified on a Ni-NTA-agarose column as described for protein expression and purification from S. cerevisiae. Purified PPTase (Supplementary Fig. 10b) was used for the KS domain activity assay.

Protein expression using a wheat germ cell-free system. PCR fragments containing the C–A–PCP domain (1–3,360 nt) or KS domain (3,361–4,809 nt) of TASI were amplified from the cDNA of M. oryzae that was cultured with 1% DSMO via PCR with primers pEU_CAT-F and pEU_CAT-R for the KS–C–A–PCP domain and pEU_F and pEU_KS_DN-R for the KS domain. PCR products were inserted into the pEU01 vector (Cell-Free Science, Matsuyama, Japan) amplified with primers pEU01-F and pEU01-R using the In-Fusion HD cloning system (Clontech). The resulting expression plasmids were designated pEU01_C-His_CAT and pEU01_KS_DN. His-tagged C–A–PCP domains and N-terminally 6×His-tagged KS domain of TASI, respectively. Cell-free protein synthesis with wheat embryo extracts was performed with a WEPROT7240 Expression kit (Cell Free Science) using pEU01_C-His_CAT and pEU01_KS_DN in 226 μl bayer format on 96-well flat-bottom plates (Iwaki) according to the manufacturer's instructions. The solubility of the translation product was confirmed with sodium dodecyl sulphate-polyacrylamide gel electrophoresis64. The reaction mixture was centrifuged at 13,000g for 30 min at 4°C, and the supernatant was used directly for the TeA biosynthesis assay.

In vitro enzyme assay. A TASI activity assay was conducted at 25°C for 2 h in 100 μl reaction mixture containing 0.5 μM TASI, 50 mM Tris-HCl (pH 7.5), 10 mM Tris (2-carboxyethyl) phosphine (TCEP), 1 mM ATp, 5% glycerol, 1 mM isoicinole and 1 mM acetyl-CoA. Acetyl-CoA (1 mM) and malonyl-CoA (1 mM) were used as equivalents of acetyl-CoA and isoicinole, respectively. The pH of the reaction mixture was determined with a pH meter (Kyowa, Japan) and the measured pH was adjusted to 7.5 using 1 M KOH. Reaction mixture was assayed at 37°C.

The reaction mixture was assayed for 1 h and the TASI activity assay was conducted in 260 μl reaction mixture. The first reaction was conducted with 0.8 μM purified MoPPT containing 100 μl soluble crude proteins containing C-His-
tagged C–A–PCP, N-his-tagged KS protein, or both, 80 μM CoA, 4 mM ATP, 2 mM MgCl₂, 8 mM TCEP, 5% glycerol and translation buffer with the WFRP7240 Expression kit (Cell Free Science) at 30 °C for 1 h. Then, 0.4 mM nucleosil and acetocetyl-CoA were added and reacted at 25 °C for 5 h. For analysis of the cyclization capability of the KS domain, the enzyme reaction was conducted in 200 μl reaction mixture containing five times-concentrated and buffer (50 mM MES (pH 6.5))-exchanged 100 μl of C–A–PCP or N-His-tagged soluble crude proteins synthesized with the cell-free system, 5% glycerol, 5 mM TCEP and 0.25 mM N-acetoxy-3-acetoacetyl-3-Il-SNAC at 25 °C for 2 h. For analysis of the condensation capability of the TASI, the enzyme reaction was conducted in the same manner as cyclization capability analysis, but using 0.25 mM 3-Il-SNAC and acetocetyl-CoA of 5% N-acetoxy-3-acetoacetyl-3-Il-SNAC.

After the enzyme reaction, the reaction mixtures were extracted with four volumes of ethanol, evaporated with an N₂ stream, and finally dissolved in 200 μl methanol for UPLC/MS analysis of TeA production. After injection of the extracted sample into a column equilibrated with 5% solvent B, the column was developed with a linear gradient from 5 to 100% solvent B over 3.0 min and kept at 100% solvent B for another 2 min. Mass spectra were collected in the electrospray ionization-positive and electrospray ionization-negative modes.

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After the enzyme reaction, the reaction mixtures were extracted with four volumes of ethanol, evaporated with an N₂ stream, and finally dissolved in 200 μl methanol for UPLC/MS analysis of TeA production. After injection of the extracted sample into a column equilibrated with 5% solvent B, the column was developed with a linear gradient from 5 to 100% solvent B over 3.0 min and kept at 100% solvent B for another 2 min. Mass spectra were collected in the electrospray ionization-positive and electrospray ionization-negative modes.
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Author contributions
C.-S.Y. wrote the manuscript. C.-S.Y. and T.M. performed biochemical and genetic experiments and bioinformatic analysis. C.-S.Y. and T.M. designed the research protocol. H.O. integrated all research.

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