Identification of a regiospecific S-oxygenase for the production of marasmin in traditional medicinal plant *Tulbaghia violacea*

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Received April 8, 2022; accepted June 19, 2022 (Edited by T. Koeduka)

Abstract

Marasmin [S-(methylthiomethyl)-L-cysteine-4-oxide] is a pharmaceutically valuable sulfur-containing compound produced by the traditional medicinal plant, *Tulbaghia violacea*. Here, we report the identification of an S-oxygenase, TvMAS1, that produces marasmin from its corresponding sulfide, S-(methylthiomethyl)-L-cysteine. The amino acid sequence of TvMAS1 showed high sequence similarity to known flavin-containing S-oxygenating monooxygenases in plants. Recombinant TvMAS1 catalyzed regiospecific S-oxygenation at S4 of S-(methylthiomethyl)-L-cysteine to yield marasmin, with an apparent *K*ₘ value of 0.55 mM. TvMAS1 mRNA accumulated with S-(methylthiomethyl)-L-cysteine and marasmin in various organs of *T. violacea*. Our findings suggest that TvMAS1 catalyzes the S-oxygenation reaction during the last step of marasmin biosynthesis in *T. violacea*.

Key words: biosynthesis, flavin-containing monooxygenase, marasmin, S-oxygenation, *Tulbaghia violacea*.

Introduction

Plants in the genus *Tulbaghia* of the Amaryllidaceae family, especially *Tulbaghia violacea* and *T. alliacea*, have been used as traditional herbal medicines to treat diseases, such as asthma, tuberculosis, rheumatism, paralysis, and hypertension in southern Africa (Aremu and Van Staden 2013; Styger et al. 2016). One of the phytochemical features of the genus *Tulbaghia* is the production of an S-substituted L-cysteine sulfoxide, marasmin [S-(methylthiomethyl)-L-cysteine-4-oxide], which is a major sulfur-containing secondary metabolite (Kubec et al. 2002, 2013). When plant tissues are disrupted, marasmin is hydrolyzed by endogenous C–S lyase to form an unstable thiosulfinate, marasmicin [S-(methylthiomethyl) (methylthio)methane-thiosulfinate] (Jacobsen et al. 1968; Kubec et al. 2002). The mechanism for the C–S lyase-catalyzed hydrolysis of marasmin is analogous to that of S-alk(en)yl-L-cysteine sulfoxides in plants of the genus *Allium* in the Amaryllidaceae family, which probably reflects the close genetic relationship between *Tulbaghia* and *Allium* (Gmelin et al. 1976; Kubec et al. 2002). Owing to its unstable nature, marasmicin spontaneously decomposes into various sulfur-containing compounds such as 2,4,5,7-tetrathiaoctane, 2,4,5,7-tetrathiaoctane-2,2-dioxide, 2,4,5,7-tetrathiaoctane-4,4-dioxide, and 2,4,5,7-tetrathiaoctane-2,2,7,7-tetraoxide (Kubec et al. 2002). The resulting compounds possess antimicrobial, antifungal, and antithrombotic activities (Block et al. 1994; Kubota et al. 1994a, 1994b; Lim et al. 1998, 1999; Ranglová et al. 2015). In addition, some sulfur-containing compounds produced during marasmin decomposition, such as (methylthiomethyl) mercaptan and bis(methylthiomethyl)sulfides, are assumed to be the cause of the odor produced upon tissue disruption in *Tulbaghia* plants (Kubec et al. 2002).

Recent biosynthetic studies suggest that the biosynthesis of sulfoxide compounds from their corresponding sulfides generally take place in plants via S-oxygenation mediated by flavin-containing monooxygenases (FMOs). In general, FMOs catalyze the transfer of one atom of molecular O₂ to a low-molecular-weight substrate using flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide phosphate.
(NADPH) as a prosthetic group and cofactor, respectively (Schlaich 2007). Based on the similarity of the amino acid sequences, plant FMOs fall into three clades. The members of each clade likely possess specialized functions for the synthesis of natural products. A clade I FMO in Arabidopsis thaliana, FMO1, catalyzes the N-hydroxylation of pippecolic acid to yield N-hydroxypippecolic acid, which induces systemic acquired resistance to bacterial and oomycete infections (Hartmann et al. 2018). The FMO members in clade II, known as YUCCA proteins, convert indole-3-pyruvic acid to auxin indole-3-acetic acid via oxidative decarboxylation (Mashiguchi et al. 2011). In contrast, clade III includes members that catalyze S-oxygenation of sulfide compounds to form the corresponding sulfoxide compounds: FMO GS-ox proteins in A. thaliana S-oxygenate S-methylthioalkyl glucosinolates for the modification of side chains (Hansen et al. 2007; Kong et al. 2016; Li et al. 2008), and AsFMO1 in garlic (Allium sativum) S-oxygenates S-allyl-L-cysteine in the biosynthesis of S-allyl-L-cysteine sulfoxide (alliin) (Yoshimoto et al. 2015). These observations support the hypothesis that marasmin is produced from its corresponding sulfide, S-(methylthiomethyl)-L-cysteine (SMTMC), via S-oxygenation by a specific clade III FMO in T. violacea. Since SMTMC possesses two sulfur atoms, an S-oxygenase showing high regiospecificity is likely involved.

In this study, we cloned the T. violacea cDNA TvMAS1, which encodes a clade III FMO. Recombinant TvMAS1 catalyzed the regiospecific S-oxygenation of SMTMC to yield marasmin. Together with the biochemical features of TvMAS1, the co-accumulation of mRNA TvMAS1 yield marasmin. Together with the biochemical features of SMTMC, the hypothesis that marasmin is produced from its corresponding sulfide, S-(methylthiomethyl)-L-cysteine (SMTMC), via S-oxygenation by a specific clade III FMO in T. violacea. Since SMTMC possesses two sulfur atoms, an S-oxygenase showing high regiospecificity is likely involved.

In this study, we cloned the T. violacea cDNA TvMAS1, which encodes a clade III FMO. Recombinant TvMAS1 catalyzed the regiospecific S-oxygenation of SMTMC to yield marasmin. Together with the biochemical features of TvMAS1, the co-accumulation of TvMAS1 mRNA with SMTMC and marasmin in T. violacea plants at the organ level suggests that marasmin is biosynthesized from SMTMC via S-oxygenation by TvMAS1.

Materials and methods

Plant materials and reagents

Bulbs of T. violacea were purchased from Soramimi Herb Shop (Shimane, Japan). Plants were grown in pots filled with vermiculite moistened with a diluted commercial nutrient solution (Hyponex, Osaka, Japan) at 25°C under a 16-h light/8-h dark photoperiod. SMTMC was purchased from FCH Group (Chernivig, Ukraine). All other chemicals were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA), Nacalai Tesque, Inc. (Kyoto, Japan), and FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

cDNA cloning from T. violacea

Total RNA was extracted from T. violacea leaves using the RNeasy Plant Mini Kit (Qiagen, Venlo, Netherlands) and treated with DNase I (Takara Bio, Shiga, Japan). Reverse transcription (RT) was performed using SuperScript VILO cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. Using the first-strand cDNA as the template, partial cDNAs corresponding to the three contigs found in the in-house RNA-sequencing data of root, bulb, leaf, and stem tissues of T. violacea (Yoshimoto et al. unpublished work), i.e., contig00043009, contig00045562, and contig00049473, were amplified by PCR using KOD-Plus DNA polymerase (Toyobo, Osaka, Japan) and the primers designed based on the sequences of these contigs (Supplementary Table S1). 5′-Rapid amplification of cDNA ends (5′-RACE) was performed using the SMARTer RACE cDNA amplification kit (Takara Bio) and the primers described in Supplementary Table S1. cDNA fragments containing the full-length coding region of TvMAS1 and TvMAS2 were isolated by RT-PCR using KOD-Plus DNA polymerase (Toyobo) and the primers described in Supplementary Table S1. The amplified fragments were cloned into pGEM-T Easy vector (Promega, Madison, WI, USA) and fully sequenced.

The nucleotide sequences identified in this study have been submitted to the DNA Data Bank of Japan (DDBJ) under the following accession numbers: TvMAS1, LC699627; TvMAS2, LC699628.

Heterologous expression of TvMAS1 in yeast

The coding region of TvMAS1 was amplified by PCR using KOD-Plus DNA polymerase (Toyobo) and the primers TvMAS-FKnpl3A and TvMAS-stop-RXhol to generate KpnI and Xhol restriction sites at the beginning and end of the coding region of TvMAS1, respectively. The nucleotide sequences of the primers used are listed in Supplementary Table S2. The amplified fragments were cloned into pGEM-T Easy vector (Promega) and was fully sequenced. The KpnI-Xhol fragment of TvMAS1 cDNA was excised and inserted between the KpnI and Xhol sites of the yeast expression vector pYES2 (Thermo Fisher Scientific). The resulting plasmid, pYES2-TvMAS1, and the pYES2 empty vector were transferred into Saccharomyces cerevisiae BJ2168 (MATa, prb1-1122, prc1-407, pep4-3, leu2, trpl, ura3-52, gal2; Nippon Gene, Tokyo, Japan) using the lithium acetate method (Gietz et al. 1992). Transformants were selected on synthetic dextrose minimal medium (Sherman 1991) lacking uracil. Induction of TvMAS1 recombinant protein expression in yeast and extraction of yeast crude protein for the biochemical assay were performed according to a previously described method (Yoshimoto et al. 2015).

Assays of TvMAS1 enzyme activities

The standard assay mixture contained 1 µg µl⁻¹ yeast crude protein, 50 mM Tris-HCl (pH 7.8), 500 µM NADPH, 500 µM FAD, 1 mM DTT, 0.1 mM EDTA, and 500 µM SMTMC. Each reaction was carried out for 1 h at 30°C. For the chemical oxidation reaction, a mixture of 500 µM SMTMC and 500 µM hydrogen peroxide was incubated for 2 h at 30°C. These enzymatic and chemical reaction products were ultrafiltered using a centrifugal ultrafiltration device (Centricut Ultramini, molecular weight cut-off 10 kDa, Kurabo, Osaka, Japan) and analyzed using liquid chromatography-mass spectrometry (LC-
MS), as described below.

For kinetic analysis, enzyme assays were performed in triplicate at SMTMC concentrations ranging from 0.05–1.5 mM. The $K_\text{m}$ value was calculated using the Michaelis–Menten equation for three independent assays.

**Extraction of sulfur-containing compounds from T. violacea**

$T. violacea$ tissues were ground to a fine powder in liquid nitrogen and subsequently freeze-dried. Ten mg of the dried tissue powder was added to 1 ml of 20 mM HCl and sonicated for 15 min at 15°C. Homogenates were centrifuged at 15,000×g for 15 min. The supernatant was ultrafiltered using a centrifugal ultrafiltration device (Centricut Ultramini, molecular cut-off 10 kDa, Kurabo), and the flow-through was analyzed using LC-MS as described below.

**Analysis of sulfur-containing compounds**

Sulfur-containing compounds in the reaction products and $T. violacea$ extracts were detected using LC-MS (LC, 1260 Infinity II; MS, 6470 LC/TQ; Agilent Technologies, Santa Clara, CA, USA) in the positive ion mode using the following conditions: column, a Microsorb-MV 100-5 C18 column (5 µm, 4.6 mm×250 mm, Agilent Technologies); solvent A, 10 mM CH$_3$COONH$_4$ (pH 7.0); solvent B, 80% (v/v) acetonitrile containing 0.25% (v/v) CH$_3$COOH; gradient program (%B), 0–10 min (3%), 10–15 min (3–100%), 15–25 min (100%), 25–26 min (100–3%), 26–36 min (3%); flow rate of mobile phase, 0.7 ml min$^{-1}$. The tandem mass spectrometry (MS/MS) spectrum of marasmin was computationally predicted using CFM-ID 3.0 (Djoumbou-Feunang et al. 2019) and used to identify marasmin in the reaction products and $T. violacea$ extracts. To analyze the amount of SMTMC in $T. violacea$ extracts and the relative amount of marasmin in enzymatic extracts, the product ion of the $S$-oxygenase gene involved in marasmin biosynthesis was monitored in multiple reaction monitoring (MRM) mode. The standard curve for the quantification of SMTMC was created using authentic SMTMC.

**Semi-quantitative RT-PCR**

Total RNA extraction and cDNA synthesis were performed as described for cDNA cloning of $TvMAS1$. First-strand cDNA derived from 10 ng of total RNA was used to amplify partial cDNA for $TvMAS1$, whereas that derived from 5 ng of total RNA was used to amplify partial cDNA for the actin gene. PCR was carried out for 32 cycles for $TvMAS1$ and 24 cycles for the actin gene, where cDNAs were exponentially amplified using KOD-Plus DNA polymerase (Toyobo). The primers used are listed in Supplementary Table S3. The PCR products were separated using agarose gel electrophoresis and stained with Midori Green Xtra (Nippon Genetics, Tokyo, Japan). Signals were detected using a gel imaging system (FAS-V; Nippon Genetics).

**Phylogenetic analysis and calculation of the identity and similarity of the amino acid sequences**

A phylogenetic tree was generated using the neighbor-joining method (Saitou and Nei 1987) after alignment using ClustalW in the MEGA 7 software (Kumar et al. 2016). The identity and similarity of the amino acid sequences were calculated using the EMBoss Needle program on the EMBL-EBI website (https://www.ebi.ac.uk/Tools/psa/emboss_needle/). The accession numbers of the protein sequences used for the analyses are summarized in Supplementary Table S4.

**Results**

**Cloning of $TvMAS1$ cDNA from $T. violacea$**

As a resource for discovering genes involved in marasmin biosynthesis, we conducted RNA-sequencing analysis of root, bulb, leaf, stem, and flower tissues of $T. violacea$ and obtained 58,266 contigs (Yoshimoto et al. unpublished work). In the present study, we used the sequence collection to identify the $S$-oxygenase gene involved in marasmin biosynthesis. We found three contigs that showed high sequence similarity with known plant $S$-oxygenase genes, garlic $AsFMO1$ involved in alliin biosynthesis (Yoshimoto et al. 2015) and $Arabidopsis FMO_{GS-OX}$ genes involved in glucosinolate biosynthesis (Hansen et al. 2007; Kong et al. 2016; Li et al. 2008). These contigs were found in the RNA-sequencing data of root, bulb, leaf, and stem tissues. Based on the sequence information of these contigs, we obtained two $T. violacea$ cDNA clones by RT-PCR and 5’-RACE using RNA from $T. violacea$ leaves as a template. These two cDNA clones were designated $TvMAS1$ and $TvMAS2$, representing $Tulbaghia violacea$ marasmin synthase 1 and 2, respectively. $TvMAS1$ and $TvMAS2$ cDNAs contained a coding region of 1,410 bp encoding a 469-amino acid polypeptide. The coding sequences of $TvMAS1$ and $TvMAS2$ differed by only two nucleotides, which resulted in a difference in one single amino acid residue: residue 138, with Ser in $TvMAS1$ and Gly in $TvMAS2$. It is unclear whether $TvMAS1$ and $TvMAS2$ are two different genes generated by gene duplication or two alleles of the same gene. However, the high sequence similarity between the coding sequences of $TvMAS1$ and $TvMAS2$ suggests that the catalytic functions of the encoded proteins are almost identical. Therefore, we focused on $TvMAS1$ for further analysis. The deduced amino acid sequence of $TvMAS1$ showed 55.4% identity with that of garlic $AsFMO1$ and approximately 50% identity with those of $Arabidopsis FMO_{GS-OX}$ genes (Table 1). Phylogenetic analysis indicated that $TvMAS1$ is a member of clade III FMO, similar to garlic $AsFMO1$ and $Arabidopsis FMO_{GS-OX}$ proteins (Figure 1A), suggesting that it functions as an $S$-oxygenase.

Plant FMOs generally possess four conserved sequence motifs: the FAD-binding motif (GxGxxG),
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NADP-binding motif (GxGxxG) that is less conserved, TGY motif, and FMO-identifying sequence motif (FxGxxxHxxxY/F) (Schlaich 2007). TvMAS1 contained fully conserved FAD-binding and TGY motifs and partially conserved NADP-binding and FMO-identifying sequence motifs (Figure 1B). The FMO-identifying sequence motif in TvMAS1 possessed one amino acid residue more than that in known plant S-oxygenating FMOs (Figure 1C). The single amino acid-residue difference between TvMAS1 and TvMAS2 at residue 138 was not observed in any of these conserved motifs.

Regiospecific S-oxygenation activity of TvMAS1 on SMTMC to form marasmin

For functional characterization, crude protein extracts from yeast expressing recombinant TvMAS1 and yeast carrying an empty vector were assayed in vitro with SMTMC as a potential substrate. The presence of marasmin in these reaction products was analyzed using LC-MS in positive ion mode. Considering that the molecular weight of marasmin was 197, the ions at m/z 198 were monitored in the selective ion monitoring mode (Figure 2A). Because authentic marasmin is not commercially available, we attempted to purify marasmin from *T. violacea* following a previously reported method (Kubec et al. 2002). Both 1H nuclear magnetic resonance (NMR) and 13C NMR data indicated that the purified extract contained marasmin, although with impurities. Therefore, we used *T. violacea* extract as a control sample containing the natural form of marasmin for LC-MS (Figure 2A). In addition, we analyzed the chemical oxidation products of SMTMC using hydrogen peroxide as an oxidant (Figure 2A).

The enzymatic reaction product of TvMAS1 exhibited two significant peaks at m/z 198, with retention time of 4.3 and 5.7 min, respectively (Figure 2A). The area of the peak at 5.7 min was larger than that of the other peak. We termed the compound eluted at 4.3 min as compound 1 and that eluted at 5.7 min as compound 2. In contrast to the TvMAS1 product, the empty vector control showed

| Similarity to TvMAS1 (%) | AsFMO1 | FMO<sub>GS-0X1</sub> | FMO<sub>GS-0X2</sub> | FMO<sub>GS-0X3</sub> | FMO<sub>GS-0X4</sub> | FMO<sub>GS-0X5</sub> |
|-------------------------|--------|----------------|----------------|----------------|----------------|----------------|
| 69.5                    | 65.2   | 65.4           | 66.1           | 66.2           | 68.1           |
| 55.4                    | 50.7   | 53.2           | 51.8           | 53.9           | 55.8           |

**Table 1.** Similarity and identity of amino acid sequence between TvMAS1 and known plant S-oxygenating FMOs.
only a single peak corresponding to compound 1 (Figure 2A). These results indicated that TvMAS1 converts SMTMC into compound 2. *T. violacea* extracts exhibited a small peak for compound 1 and a large peak for compound 2 (Figure 2A). The LC-MS spectrum of the *T. violacea* extracts was similar to that of the enzymatic reaction product of TvMAS1. The chemical oxidation product of SMTMC exhibited only a single peak for compound 1 (Figure 2A).

The chemical structures of compounds 1 and 2 were analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Compound 1 generated two major product ions at *m/z* 134.99 and 88.03, while compound 2 generated two major product ions at *m/z* 150.04 and 61.01 (Figure 2B). The MS-based compound identification tool, CFM-ID 3.0 (Djoumbou-Feunang et al. 2019), predicted that the product ion at *m/z* 134.99 generated from compound 1 was C$_4$H$_7$OS$_2^+$ fragment, in which a hydroxy group was attached to the sulfur atom at the S5 position (Figure 2C, left). In contrast, the product ion at *m/z* 150.04, generated from compound 2, was predicted to be a C$_4$H$_8$NO$_3$S$^+$ fragment, in which a hydroxy group was attached to the sulfur atom at the S4 position (Figure 2C, right). Based on the structural characteristics of these product ions, compound 1 was considered as S-(methylthiomethyl)-L-cysteine-6-oxide, an isomer of marasmin with an oxygenated sulfur atom at the S6 position (Figure 2D, left), while compound 2 was identified as marasmin with an oxygenated sulfur atom at the S4 position (Figure 2D, right). These results indicated that TvMAS1 catalyzes regiospecific S-oxygenation of SMTMC to yield marasmin.

To determine the kinetic properties of TvMAS1, enzyme assays were performed in triplicates at SMTMC concentrations ranging from 0.05 to 1.5 mM. The formation of marasmin by TvMAS1 obeyed Michaelis–Menten kinetics, with an apparent $K_m$ value of 0.55 ± 0.20 mM (Figure 3).
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Discussion

Phylogenetic classification of TvMAS1

In this study, we identified a *T. violacea* gene, *TvMAS1*, that showed high sequence similarity with known S-oxygenating clade III FMOs (Table 1). *TvMAS1* was phylogenetically classified into clade III plant FMO (Figure 1A), which contains S-oxygenases responsible for the side-chain modification of aliphatic glucosinolates (Hansen et al. 2007; Kong et al. 2016; Li et al. 2008) and the biosynthesis of alliin (Yoshimoto et al. 2015). *TvMAS1* possesses four sequence motifs that are conserved in FMO proteins (Figure 1B). Interestingly, the FMO-identifying sequence motif in *TvMAS1* had one additional amino acid residue compared with those in other clade III FMOs (Figure 1C). To the best of our knowledge, the structural and functional roles of FMO-identifying sequence motifs have not yet been determined. The observation that recombinant *TvMAS1* showed S-oxygenation activity on SMTMC in vitro (Figures 2, 3) suggested that the additional amino acid residue in the FMO-identifying sequence motif at the position observed for *TvMAS1* did not result in the loss of the catalytic activity of FMO.

Regiospecific S-oxygenation activity of *TvMAS1*

SMTMC has two sulfide sulfur atoms at the S4 and S6 positions that can be oxygenated. The absence of a specific enzyme seems to trigger the formation of S-(methylthiomethyl)-L-cysteine-6-oxide, rather than marasmin, from SMTMC. When SMTMC was oxygenated with hydrogen peroxide, S-(methylthiomethyl)-L-cysteine-6-oxide, but not marasmin, was formed (Figure 2). The empty vector control reaction also formed S-(methylthiomethyl)-L-cysteine-6-oxide, but not marasmin, from SMTMC (Figure 2), indicating that the dissolved molecular oxygen predominantly attacked the sulfur atom at the S6 position. It is likely that the oxidants are more accessible to the sulfur atom at the S6 position than at the S4 position in SMTMC, which might be attributed to the difference in the distance of these two sulfur atoms to the relatively bulky carboxyl and amino groups.

In contrast, the assay using the protein extract from the yeast expressing *TvMAS1* mainly formed marasmin from SMTMC, with the apparent *K_m* value of 0.55±0.20 mM (Figures 2, 3). The amount of S-(methylthiomethyl)-L-cysteine-6-oxide generated in the *TvMAS1* assay was far lesser than that of the vector

Accumulation patterns of *TvMAS1* mRNA, SMTMC, and marasmin in *T. violacea*

Biochemical assay results of *TvMAS1* suggested that SMTMC is a biosynthetic intermediate of marasmin in *T. violacea*. Therefore, we analyzed whether *TvMAS1* mRNA is expressed in the tissues of *T. violacea* that contain SMTMC and marasmin. *T. violacea* plants were separated into four parts: roots, bulbs, white leaves, and green leaves (Figure 4A). The mRNA levels of *TvMAS1* were analyzed using semi-quantitative RT-PCR, and the SMTMC and relative marasmin levels in these tissues were analyzed using LC-MS/MS.

Although using a single primer set, *TvMAS1* cDNA was amplified with *TvMAS2* cDNA by RT-PCR because of their high sequence similarity. The results suggested that the total amounts of *TvMAS1* and *TvMAS2* mRNAs were similar in all analyzed tissues (Figure 4B). Both SMTMC and marasmin were detected in all four analyzed tissues, with a variation of <75% for the highest level for SMTMC and <65% for the highest level for marasmin (Figure 4C, D). SMTMC was particularly abundant in the roots (Figure 4C), whereas marasmin accumulated at high levels in the bulbs (Figure 4D). In the leaves, the amounts of both SMTMC and marasmin were higher in the green parts than that in the white parts (Figure 4C, D).

![Figure 4](image_url)
control (Figure 2A). These results indicated that the TvMAS1-catalyzed formation of marasmin occurred more efficiently than the non-enzymatic formation of \( S\)-(methylthiomethyl)-L-cysteine-6-oxide in the TvMAS1 assay. Interestingly, the ratio of marasmin to \( S\)-(methylthiomethyl)-L-cysteine-6-oxide in the extract of \( T. \) violacea was similar to that in the enzymatic assay of TvMAS1 (Figure 2A), suggesting that SMTMC is oxygenated mainly enzymatically by TvMAS1 to yield marasmin in \( T. \) violacea. The molecular mechanism underlying regiospecific \( S\)-oxygenation of TvMAS1 is unclear and requires further elucidation.

The primary physiological role of marasmin in \( T. \) violacea is probably the breakdown into the sulfur-containing defense compounds when tissues are damaged due to attack by natural enemies. The breakdown of marasmin is initiated by endogenized C-S lyase, which cleaves the C-S bond of marasmin to form pyruvate, sulfenic acid, and ammonia (Jacobsen et al. 1968; Kubec et al. 2002). The catalytic mechanism of C-S lyase for marasmin is considered to be the same as that of alliinase, the C-S lyase hydrolyzing \( S\)-alk(en)ylcysteine sulfoxides, such as alliin and isoalliin in \( Allium \) plants (Jacobsen et al. 1968; Kubec et al. 2002). Considering the catalytic mechanism of alliinase in \( Allium \) plants (Shimon et al. 2007), the mechanism of marasmin breakdown by the C-S lyase probably involves the abstraction of the proton attached to the chiral carbon at the C2 position of marasmin, which causes synchronous cleavage of the C-S bond between the carbon atom at the C3 position and the sulfoxide sulfur atom at the S4 position. For cleavage of the C-S bond, a positive charge on the sulfur atom at the S4 position is required. Since \( S\)-(methylthiomethyl)-L-cysteine-6-oxide does not have a proton that can be abstracted for C-S bond cleavage, it cannot be used as a substrate for the C-S lyase in \( T. \) violacea. Therefore, the regiospecific \( S\)-oxygenation activity of TvMAS1 against SMTMC is important for \( T. \) violacea in terms of efficient production of defense compounds.

**Stereospecificity of \( S\)-oxygenation by TvMAS1**

In contrast to the regiospecificity of TvMAS1, the stereospecificity of TvMAS1 remains undetermined because two diastereomers of marasmin that can be formed from SMTMC by \( S\)-oxygenation, \((R,S)\)-\( S\)-(methylthiomethyl)cysteine-4-oxide and \((R,C)\)-\( S\)-(methylthiomethyl)cysteine-4-oxide, are not commercially available.

Previously, the natural form of marasmin in \( T. \) violacea was reported to be \((R,S)\)-\( S\)-(methylthiomethyl)cysteine-4-oxide (Kubec et al. 2002). The structural arrangement around the sulfur atom of this compound is similar to that of \((R,S)\)-allylcysteine sulfoxide, the major natural form of alliin in garlic (Figure 5). Garlic AsFMO1 catalyzes the highly stereoselective \( S\)-allyl-L-cysteine to yield \((R,S)\)-allylcysteine sulfoxide (Yoshimoto et al. 2015). Because AsFMO1 and TvMAS1 share high amino acid sequence similarity (Table 1), it is likely that TvMAS1 preferentially yields \((R,R)\)-\( S\)-(methylthiomethyl)cysteine-4-oxide from SMTMC. Further studies, including chemical synthesis of \((R,R)\)-\( S\)-(methylthiomethyl)cysteine-4-oxide and \((R,S)\)-\( S\)-(methylthiomethyl)cysteine-4-oxide followed by the comparison of elution profiles of these compounds and enzymatic product of TvMAS1, are needed to determine the stereospecificity of TvMAS1 on SMTMC.

**Hypothetical biosynthetic pathway of marasmin in \( T. \) violacea**

The conversion of SMTMC into marasmin by TvMAS1 (Figures 2, 3) and the coexistence of \( TvMAS1 \) mRNA with SMTMC and marasmin in \( T. \) violacea at the organ level (Figure 4) suggest that SMTMC is a biosynthetic intermediate of marasmin in \( T. \) violacea. Considering its structural characteristics, SMTMC is likely to be biosynthesized from \( L\)-cysteine or its derivatives, such as glutathione. The hypothetical biosynthetic pathway of marasmin via SMTMC in \( T. \) violacea is shown in Figure 5. This pathway is similar to that of \( S\)-alk(en)yl-L-cysteine sulfoxides in \( Allium \) plants, such as alliin in garlic (Yoshimoto and Saito 2019; Yoshimoto et al. 2015), in terms of the biosynthetic precursor and the conversion of sulfide to sulfoxide by a specific FMO at the last step of biosynthesis (Figure 5). The apparent \( K_m \) value of TvMAS1 for SMTMC (0.55±0.20 mM; Figure 3) is comparable to that of garlic AsFMO1 for \( S\)-allyl-L-cysteine (0.25±0.11 mM; Yoshimoto et al. 2015), suggesting that these two \( S\)-oxygenation reactions proceed with similar efficiency. In addition, the plant tissues in which the \( S\)-oxygenation reaction occurs might be common between marasmin biosynthesis in \( T. \) violacea and alliin biosynthesis in garlic because

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![Figure 5](Image 323x633 to 522x734)

Figure 5. Hypothetical biosynthetic pathway of marasmin in \( T. \) violacea analogous to that of alliin in garlic. The previously proposed biosynthetic pathway for the major natural form of alliin [(\( R,S)\)-\( S\)-allylcysteine sulfoxide] in garlic (above) and the hypothetical biosynthetic pathway for the natural form of marasmin [(\( R,R)\)-\( S\)-(methylthiomethyl)cysteine-4-oxide] in \( T. \) violacea (below). In the last step, both pathways include the \( S\)-oxygenation of the sulﬁdes biosynthetic intermediate, catalyzed by a specific FMO.

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both TvMAS1 mRNA in *T. violacea* (Figure 4B) and AsFMO1 mRNA in garlic (Yoshimoto et al. 2015) were detected in roots, bulbs, and leaves. Given the close genetic relationship between *Tulbaghia* and *Allium*, it is not surprising that these two genera possess similar biosynthetic machinery. Knowledge of the biosynthesis of *S*-alk(en)yl-L-cysteine sulfoxides in *Allium* plants will help in understanding the mechanism of marasmin biosynthesis in *Tulbaghia* plants and vice versa.

In the present study, we identified TvMAS1 as a regiospecific S-oxygenase that converts SMTMC into marasmin. Our findings provide insights into the molecular mechanisms of marasmin biosynthesis, which could contribute to the molecular breeding of plants that biosynthesize valuable sulfur-containing metabolites in the future.

Acknowledgements

We thank Ryo Nakabayashi and Tetsuya Mori (RIKEN CSRS, Japan) for their technical help with metabolite analysis and valuable discussions.

Author contributions

J.W., M.K., H.T., K.S., M.Y., and N.Y. designed research; J.W., H.S., N.N., M.K., M.Y., and N.Y. performed research; J.W., H.S., N.N., M.K., H.T., K.S., M.Y., and N.Y. analyzed data; and J.W. and N.Y. wrote the paper.

Funding

This work was supported in part by JSPS KAKENHI Grant Numbers 20K07097 (to N.Y.) and 19H05652 (to K.S.), and the research grant from Kobayashi Foundation (to M.Y.).

Conflict of interest

The authors declare no competing interest.

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