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Article

TcGLIP GDSL Lipase Substrate Specificity Co-determines the Pyrethrin Composition in *Tanacetum cinerariifolium*

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ABSTRACT: Natural pesticides pyrethrins biosynthesized by *Tanacetum cinrerariifolium* are biodegradable and safer insecticides for pest insect control. TcGLIP, a GDSL lipase underpinning the ester bond formation in pyrethrins, exhibits high stereo-specificity for acyl-CoA and alcohol substrates. However, it is unknown how the enzyme recognizes the other structural features of the substrates and whether such specificity affects the product amount and composition in *T. cinrerariifolium*. We report here that the cysteamine moiety in (1*R*,3*R*)-chrysanthemoyl CoA and the conjugated diene moiety in (*S*)-pyrethrolone play key roles in the interactions with TcGLIP. CoA released from chrysanthemoyl CoA in the pyrethrin-forming reaction reduces the substrate affinity for TcGLIP by feedback inhibition. (*S*)-Pyrethrolone shows the highest catalytic efficiency for TcGLIP, followed by (*S*)-cinerolone and (*S*)-jasmololone, contributing, at least in part, to determine the pyrethrin compositions in *T. cinerariifolium*.

KEYWORDS: *pyrethrins, GDSL esterase/lipase, substrate specificity, feedback inhibition*

INTRODUCTION

Pyrethrins are natural insecticides produced by *Tanacetum cinerariifolium*. They consist of pyrethrin I/II, cinerin I/II, and jasmolin I/II, resulting from the esterification of (1*R*,3*R*)-chrysanthemic and (1*R*,3*R*)-pyrethric acids with (*S*)-pyrethrolone, (*S*)-cinerolone, and (*S*)-jasmololone, which are collectively referred to as rethrolones.[1,2]

It has been described to date that the primary target of pyrethrins and pyrethroids are voltage-sensitive sodium channels involved in the nerve impulse conduction.[3] The insecticides keep sodium channels open for a prolonged time, inducing repetitive firing or conduction block in the nervous system, thereby causing knockdown and death of insects.[4−6] Recently, pyrethrins have been found to modulate odorant receptors expressed in antennae to exhibit repellency against mosquitoes[7] and fruit flies.[8]

Compared to pyrethrins, synthetic pyrethroids are employed more intensively for pest control.[9,10] However, pyrethrins (or *Tanacetum* flower extract "pyrethrum") are still used to control household and agricultural pests, as they are safer and environmentally benign.[2] In addition, they are less affected by the knockdown resistance (kdr) mutations than synthetic pyrethroids in mosquitoes,[11] spurring the demand. Hence, several groups have explored enzymes involved in their biosynthesis.[1,2,12,13] By administering ^{13}C-labeled glucose, we showed that the acid and alcohol moieties of pyrethrins are biosynthesized by the non-mevalonate and oxylipin pathways, respectively.[1,14] Also, we found with labeled precursors that pyrethrins are biosynthesized through *cis*-jasmone, unlike jasmonic acid produced via a distinct pathway.[15] Studies showed that the cyclopropane ring is formed by chrysanthemol diphosphate synthase,[16] and the diphosphate is hydrolyzed by either chrysanthemol diphosphate synthase[17] or a Nudix family hydrolase.[18] The methoxycarbonyl moiety in type II pyrethrins is formed by P450 oxidoreductase in conjunction with aldehyde hydrogenases and a methyltransferase.[19]

On the other hand, *cis*-jasmone hydroxylation and the side chain of pyrethrolone are catalyzed by P450 oxidases.[20,21] We found that TcGLIP, a GDSL esterase/lipase protein (GELP), synthesizes pyrethrins using the CoA thioester of chrysanthemic/pyrethric acid and rethrolones in the last biosynthetic step[22] (Figure 1). TcGLIP is expressed in flowers and leaves of *T. cinerariifolium* and serves as a principal means of defense against herbivore attacks.[22] Therefore, TcGLIP is as vital for *T. cinerariifolium* as is carbonic anhydrase underpinning photosynthesis.[23,24]

TcGLIP has high substrate specificity with regard to recognizing the absolute configurations of the acid and alcohol moieties.[22] However, it remains unknown how other structural features of the substrates affect the ester-forming reaction of TcGLIP, resulting in the pyrethrin amount and composition in *T. cinerariifolium*. Hence, this study aims to clarify whether and how thioesters of (1*R*,3*R*)-chrysanthemic acid and (*S*)-rethrolones and their analogs interact with TcGLIP.

METHODS

Synthesis of Substrates. All of the purchased chemicals were used without purification. Nuclear magnetic resonance (NMR), infrared (IR), High resolution-mass spectrometry (HR-MS), and optical rotation of the compounds were obtained by an Avance III 400 spectrometer (400 MHz, Bruker Biospin, Germany), IRAffinity-1

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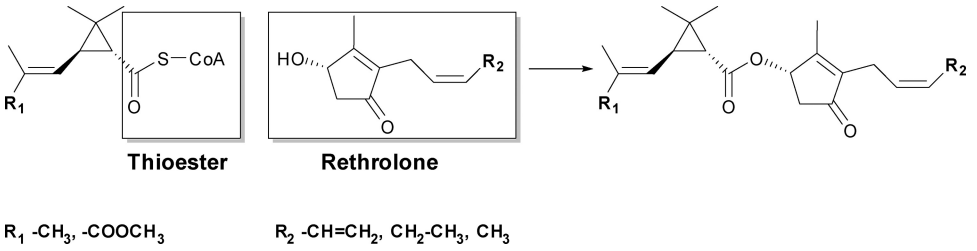

Figure 1. Reaction catalyzed by TcGLIP, a GDSL esterase/lipase expressed in *T. cinerariifolium*.

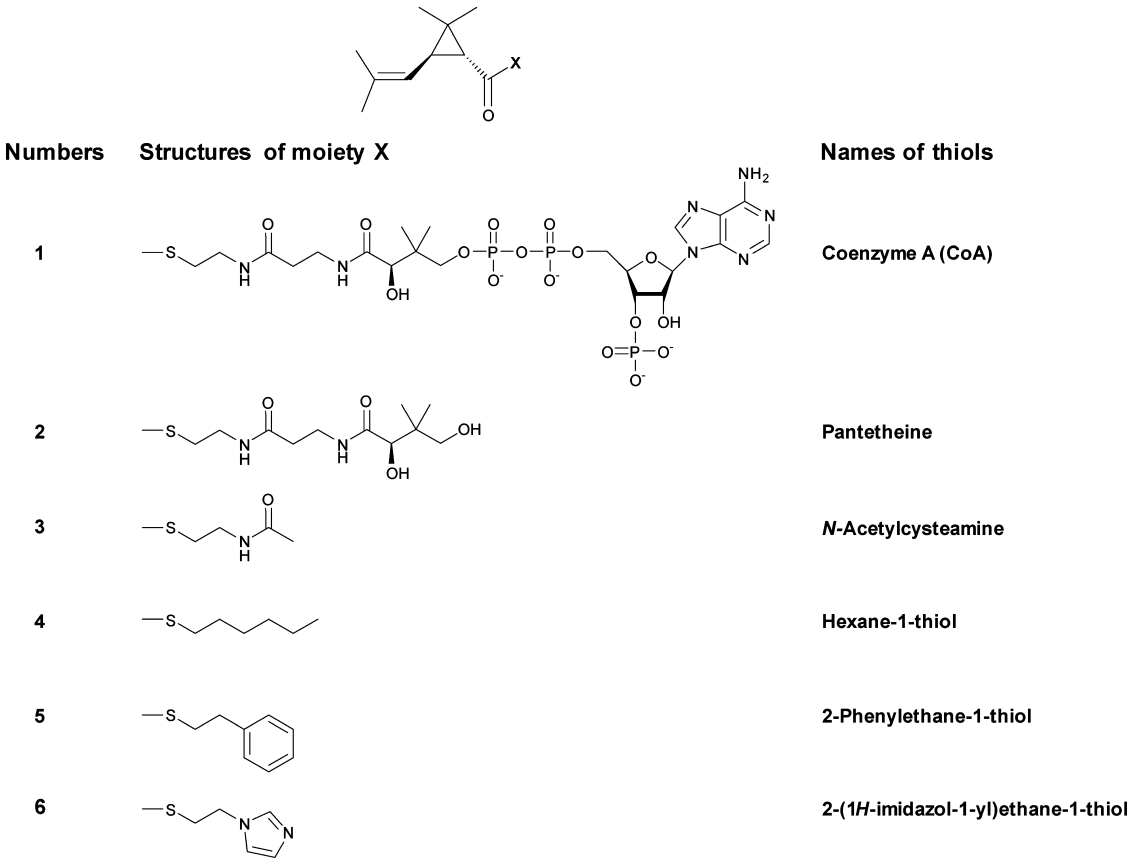

Figure 2. Structures of thioesters of (1*R*,3*R*)-chrysanthemic acid used as substrates for the pyrethrin-forming reaction catalyzed by TcGLIP.

(Shimadzu, Japan), Q-Tof Premier (Waters, U.K.), and SEPA-300 (Horiba, Japan), respectively. (1*R*,3*R*)-Chrysanthemoyl CoA (**1**) was synthesized as reported.[22]

S-(1*R*,3*R*)-Chrysanthemoyl pantetheine (**2**). To an aqueous solution of 130 mg of pantethine (235 μmol, MilliporeSigma, USA) in 5 mL of distilled water, 500 mg of $NaHCO_3$ and 0.4 mg of dithiothreitol (DTT) were added and stirred for 15 min at room temperature, then 100 mg (460 μmol) of (1*R*,3*R*)-chrysanthemoyl imidazolide[22] in 20 mL tetrahydrofuran (THF) was added dropwise at room temperature, and finally the reaction mixture was stirred for 3 h. After evaporation, the residue was purified by silica gel flash chromatography (Biotage, Sweden) with a chloroform−methanol mixture (90:10) to yield 39.0 mg (23.3 μmol) of *S*-(1*R*,3*R*)-chrysanthemoyl pantetheine (**2**) (yield 9.93%) (Figure 2).

S-(1*R*,3*R*)-Chrysanthemoyl *N*-acetylcysteamine (**3**). To a solution of 27.3 mg (229 μmol) of *N*-acetylcysteamine (MilliporeSigma) and equimolar imidazole in 10 mL of THF, equimolar (1*R*,3*R*)-chrysanthemoyl imidazolide was added at room temperature, and the reaction mixture was stirred for 18 h. After evaporation, the resultant reaction mixture was purified by silica gel flash chromatography (Biotage) using a hexane−ethyl acetate solvent system (linear gradient from hexane 100% to ethyl acetate 100%) to yield 19.8 mg of compound **3** (73.4 μmol, yield 32.1%).

S-(1*R*,3*R*)-Chrysanthemoyl hexane-1-thiol (**4**). To a solution of 54.2 mg (558 μmol) of hexane-1-thiol (MilliporeSigma) and equimolar imidazole in THF, equimolar (1*R*,3*R*)-chrysanthemoyl imidazolide (100 mg) was added and stirred for 18 h. The products were purified by silica gel flash column chromatography as performed for compound **3**, affording 49.0 mg of compound **4** (183 μmol, yield 32.8%).

S-(1*R*,3*R*)-Chrysanthemoyl phenylethane-1-thiol (**5**) was synthesized similarly to compound **4** using 558 μmol of 2-phenylethanethiol, imidazole, and (1*R*,3*R*)-chrysanthemoyl imidazolide. Silica gel flash column chromatography was employed to purify the compound as described for compounds **3** and **4**, affording 141.0 mg of compound **5** (489 μmol, yield 87.7%).

S-(1*R*,3*R*)-Chrysanthemoyl 2-(1*H*-imidazol-1-yl)ethane-1-thiol (**6**). Ethylene sulfide (0.52 mL, 8.8 mmol) and imidazole (1.8 g, 26.4 mmol) were reacted in toluene to obtain 2-(1*H*-imidazol-1-yl)ethane-1-thiol according to the literature.[25] The reaction mixture was filtered, and the filtrate was evaporated. The residue (226.6 mg) was reacted with 186.8 mg of (1*R*,3*R*)-chrysanthemic acid imidazolide (857 μmol) in THF for 2 h at room temperature. After evaporation,

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the residue was purified by silica gel chromatography with a chloroform−methanol mixture (96:4), affording 80.6 mg of compound **6** (289 μmol, yield 33.7%).

(*S*)-Pyrethrolone (**7**), (*S*)-jasmololone (**8**), and (*S*)-cinerolone (**9**) (Figure 3) were obtained by hydrolysis of pyrethrin I, jasmolin I, and

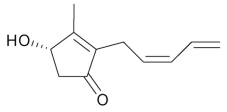
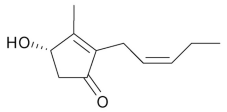
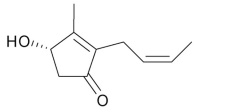
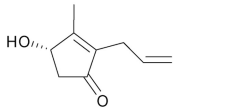
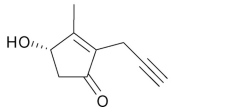
Numbers	Structures	Names of alcohols
7		(*S*)-Pyrethrolone
8		(*S*)-Jasmololone
9		(*S*)-Cinerolone
10		(*S*)-Allethrolone
11		(*S*)-Propargyllone

Figure 3. Structures of rethrolones used as alcohol substrates for the pyrethrin-forming reaction catalyzed by TcGLIP.

cinerin I, respectively, with twofold equimolar amounts of potassium carbonate in methanol. The reaction products were purified first by silica gel column chromatography with a chloroform−methanol mixture (9:1) and then with a semi-preparative chiral column (CHIRALPAK-IA, Daicel, Japan) using a hexane-2-propanol mixture (97:3) at a flow rate of 5 mL min^{-1}. In this work, 25.6 mg of (*S*)-pyrethrolone (132.4 μmol, yield 28.6%), 28.3 mg of (*S*)-jasmololone (157.2 μmol, yield 37.7%), and 10.9 mg of (*S*)-cinerolone (66.7 μmol, yield 14.3%) were obtained from 125 mg of pyrethrin I (462.8 μmol), 138.0 mg of jasmolin I (417.4 μmol), and 146 mg of cinerin I (460.6 μmol), respectively. Synthetic alcohol substrates, (*S*)-allethrolone (**10**) and (*S*)-propargyllone (**11**), supplied by Dainihon Jochugiku Co., were purified with the CHIRALPAK-IA column with a hexane-2-propanol mixture (97:3) at a flow rate of 5 mL min^{-1}.

Purity of all compounds was >95% as measured by high-performance liquid chromatography (HPLC) (Supporting Information, Figures S1 and S2). The specific rotation and MS and NMR data of compounds **2**−**6** are shown in Supporting Information Tables S1−S5 and Figures S3−S7.

Enzyme Assay. TcGLIP (gene accession number JN418994) was expressed using the pMAL-c4E vector (New England Biolabs, USA) as fusion with the maltose-binding protein (MBP) by the *Escherichia coli* Origami B strain (MilliporeSigma) and tested for determination of kinetic parameters of substrates since MBP had no substantial effect on the interactions with substrates.[26] The MBP fusion of TcGLIP, which is referred to as "TcGLIP" in the following sections, was purified using an amylose resin (New England Biolab) and a Mono Q column (10/100 GL, Cytiva, Sweden), as previously described.[22] The reaction assays were performed in a 100 μL mixture containing 200 or 250 ng of TcGLIP and various concentrations of substrates in 50 mM Tris-HCl (pH 7.5). The kinetic parameters (K_m and V_{max}) for the thioester substrates (compounds **1**−**6**) were determined in the presence of 1 mM (*S*)-pyrethrolone (**7**), while those for the alcohol substrates (compounds **7**−**11**) were obtained in the presence of 2 mM (1*R*,3*R*)-chrysanthemoyl CoA (**1**). The reaction mixture was incubated for 10 min at 25 °C, and the reaction was stopped by adding 10 μL of acetic acid. The reaction was then extracted using 100 μL of hexane, and 10 μL of the extract was subjected to HPLC analyses. HPLC was performed using an Agilent 1200 HPLC system (Agilent technologies, USA) using a Cadenza CD-C18 column (4.6 × 100 mm; Imtakt, Japan) with ultraviolet (UV) detection at 230 nm and a 80% acetonitrile−20% water mixture as the solvent at a flow rate of 1.0 mL min^{-1}, except for detection of the ester formed by (1*R*,3*R*)-chrysanthemoyl CoA and propargyllone (**11**), where a 70% acetonitrile−30% water mixture was used.

To evaluate the effects of inhibitors on TcGLIP, it was preincubated for 10 min with the inhibitors at 25 °C and then reacted with the substrates for 10 min at 25 °C. The resultant products were analyzed as described above.

Data Fitting. The kinetic data were fitted by nonlinear regression according to the Michaelis−Menten equation, and the Lineweaver−Burk plots were fitted by linear regression using Prism (GraphPad Software, USA).

Quantification of Pyrethrins in *T. cinerariifolium*. The buds frozen in liquid nitrogen were minced in 5 mL of acetone and kept at room temperature for 2 h. The acetone extract (0.5 mL) was filtered with a 0.45 μm filter and subjected to HPLC analysis. Pyrethrin I/II, cinerin I/II, and jasmolin I/II were monitored at 230 nm using a Cosmosil 3PBr column (3.0 x 100 mm, nacalai tesque, Japan) with a 65% acetonitrile−35% ammonium acetate (50 mM) mixture at a flow rate of 0.6 mL min^{-1}.

Phylogenetic Analysis. The phylogenetic tree for *Arabidopsis thaliana* GELPs and TcGLIP was constructed by Geneious (Biomatters, New Zealand) as follows. Amino sequences of these

Table 1. Kinetic Parameters of Substrates for the Pyrethrin-Forming Activity of TcGLIP[a]

substrates	K_m (μM)	V_{max} (nkat mg $protein^{-1}$)	V_{max}/K_m (nkat mg $protein^{-1}$ mM^{-1})
1	520 ± 95	2.34 ± 0.15	4.49
2	208 ± 22	1.71 ± 0.06	8.25
3	100 ± 33	1.81 ± 0.18	18.0
4	NR[b]		
5	NR[b]		
6	107 ± 6[c]	3.33 ± 0.09[c]	31.1[c]
7	376 ± 56	2.57 ± 0.13	6.83
8	358 ± 167	0.78 ± 0.12	2.17
9	822 ± 186	1.67 ± 0.17	2.04
10	>2000[d]	~1.86[d]	ND[d]
11	>2000[d]	~1.49[d]	ND[d]

[a]Data are represented as the mean ± standard error (n = 3 or 6). The kinetic parameters for compounds **1**−**6** were determined with 1 mM (*S*)-pyrethrolone (**7**), whereas those for compounds **7**−**11** were determined with 2 mM (1*R*,3*R*)-chrysanthemoyl CoA (**1**). [b]No ester-forming reaction was observed. [c]The kinetic parameters were determined using a separate TcGLIP preparation (Supporting information Figure S8). [d]Could not be determined because the specific activity did not plateau.

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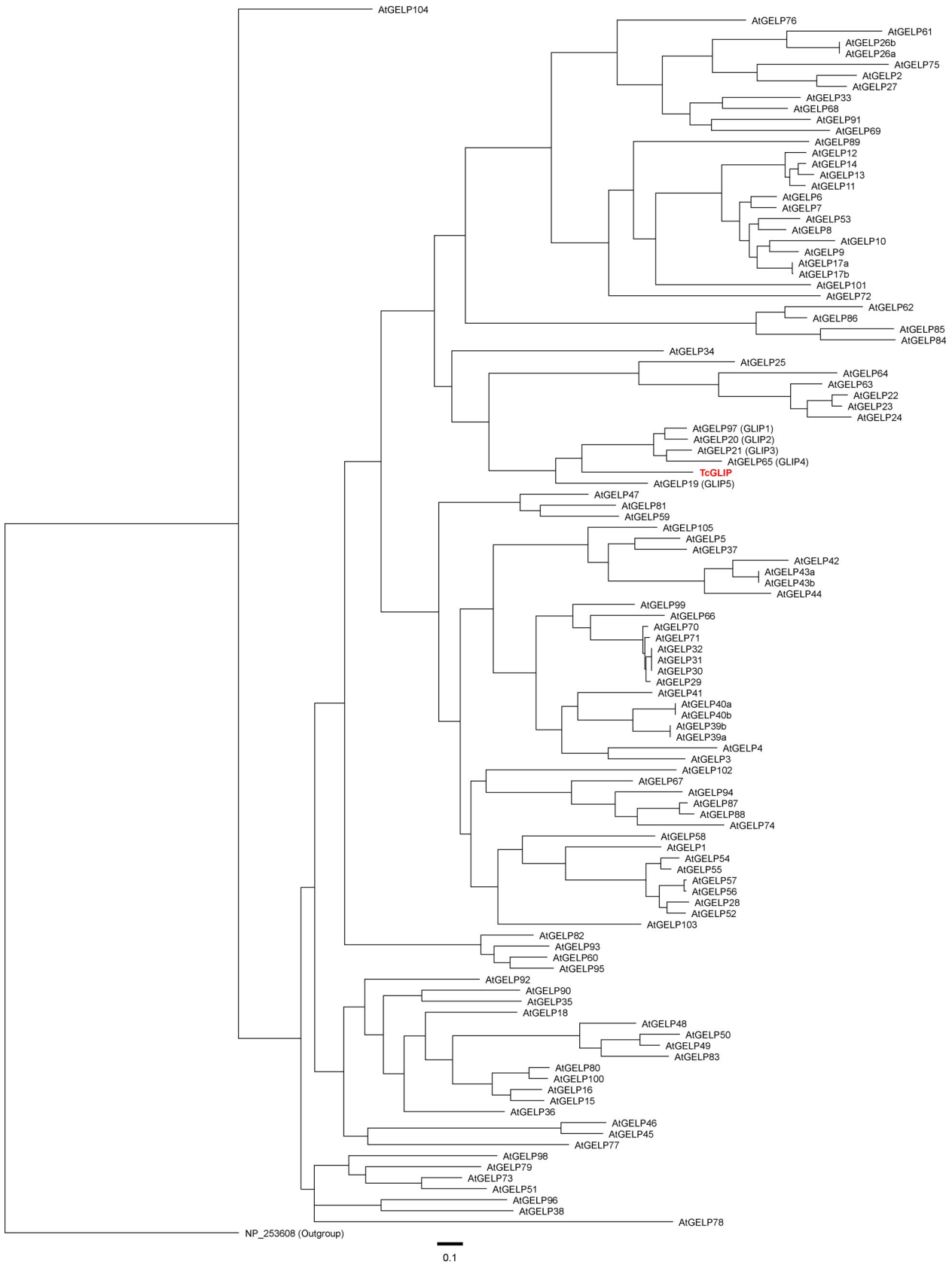

Figure 4. Phylogenetic tree of *A. thaliana* GELPs and TcGLIP constructed with *P. aeruginosa* (NP_253608) as the outgroup. TcGLIP was related to a subclade consisting of *A. thaliana* GLIP1-5. Horizontal bar represents 0.1 nucleotide substitution per site.

proteins and a related enzyme from *Pseudomonas aeruginosa* (NP_253608) as the outgroup were aligned using MAFFT,[27,28] and the alignment was cleaned by trimAl.[29] The phylogenetic tree was constructed by raxmlGUI with 100 Bootstrap analyses.[30]

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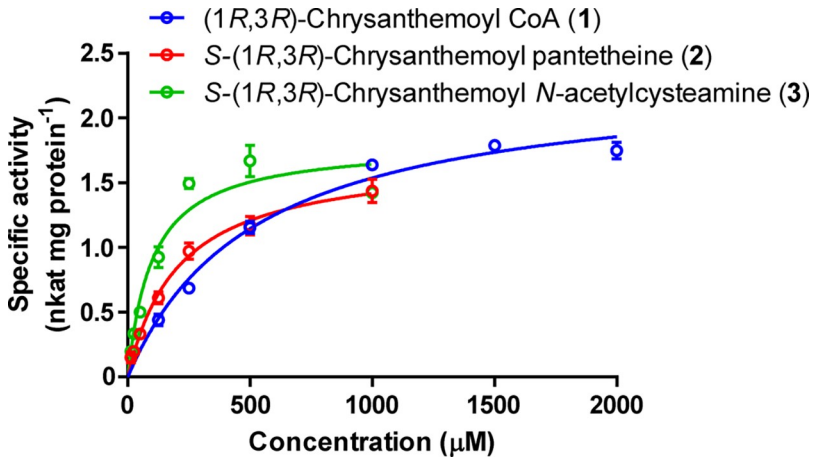

Figure 5. Concentration−catalytic activity relationships of thioester substrates **1**−**3** for the pyrethrin I-forming activity of TcGLIP. Each data plot represents the mean ± standard error of the mean (n = 6). Compounds **2** and **3** were not tested at concentrations higher than 1 mM because of their limited solubility in the buffer.

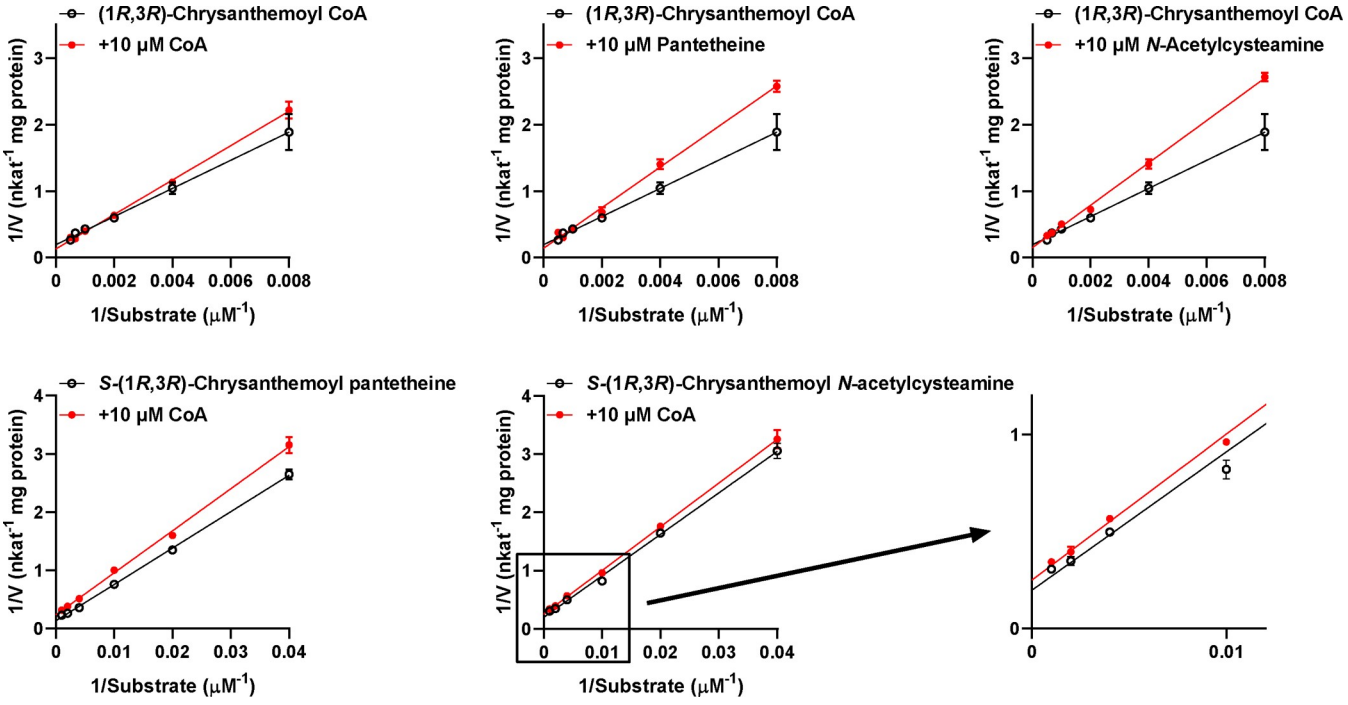

Figure 6. Effects of 10 μM inhibitors (CoA, pantetheine, and N-acetylcysteamine) on the pyrethrin I-forming reaction of (1R,3R)-chrysanthemoyl CoA (**1**), S-(1R,3R)-chrysanthemoyl pantetheine (**2**), and S-(1R,3R)-chrysanthemoyl N-acetylcysteamine (**3**) with (*S*)-pyrethrolone (**7**) at TcGLIP. In the Y axis, V is the specific activity. Each data plot represents the mean ± standard error of the mean (n = 3).

RESULTS AND DISCUSSION

TcGLIP is unique among plant GELPs in that it is the only GELP that synthesizes the secondary metabolites acting directly on herbivores.[31] A phylogenetic tree revealed that TcGLIP is similar to *A. thaliana* GLIP1-5 (Figure 4), implying that these *A. thaliana* enzymes underlie defenses as reported.[32−34] Some or all of these enzymes may catalyze ester-bond formation, as in the case of tomato GDSL1.[35] Hence, it is of value in the future to compare the function and structure−substrate interactions of the TcGLIP-related GELPs for elucidating their roles in maintaining plant homeostasis.

This study has investigated whether synthetic and natural compounds (Figures 2 and 3) work as substrates of TcGLIP in the pyrethrin-forming reaction. We found that TcGLIP utilized not only (1R,3R)-chrysanthemoyl CoA (**1**) but also (1R,3R)-chrysanthemoyl N-acetylcysteamine (**2**) and pantetheine (**3**) as substrates (Figure 5). However, it did not recognize (1R,3R)-chrysanthemic acid thioesters of hexane-1-thiol (**4**) and 2-phenylethane-1-thiol (**5**) (Table 1), indicating that the thiol and the neighboring ethylene ($-CH_2-CH_2-$) moiety and double bond alone are insufficient for the interactions with TcGLIP as substrates.

TcGLIP interacted with S-(1R,3R)-chrysanthemoyl 2-(1*H*-imidazol-1-yl)ethane-1-thiol (**6**) to yield pyrethrin I (Supporting information: Concentration−specific activity relationship for compound **6** obtained using a different TcGLIP preparation, Figure S8). Hence, the ability of the carbonyl group to form a hydrogen bond, which the imidazole C═N bond also has, is critical for the interactions with TcGLIP.

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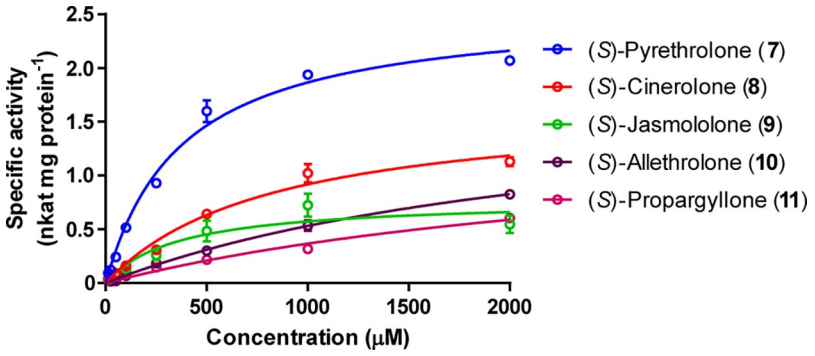

Figure 7. Concentration−catalytic activity relationships of natural and synthetic alcohol substrates for the pyrethrin I-forming activity of TcGLIP. Each data plot represents the mean ± standard error of the mean (n = 3 or 6).

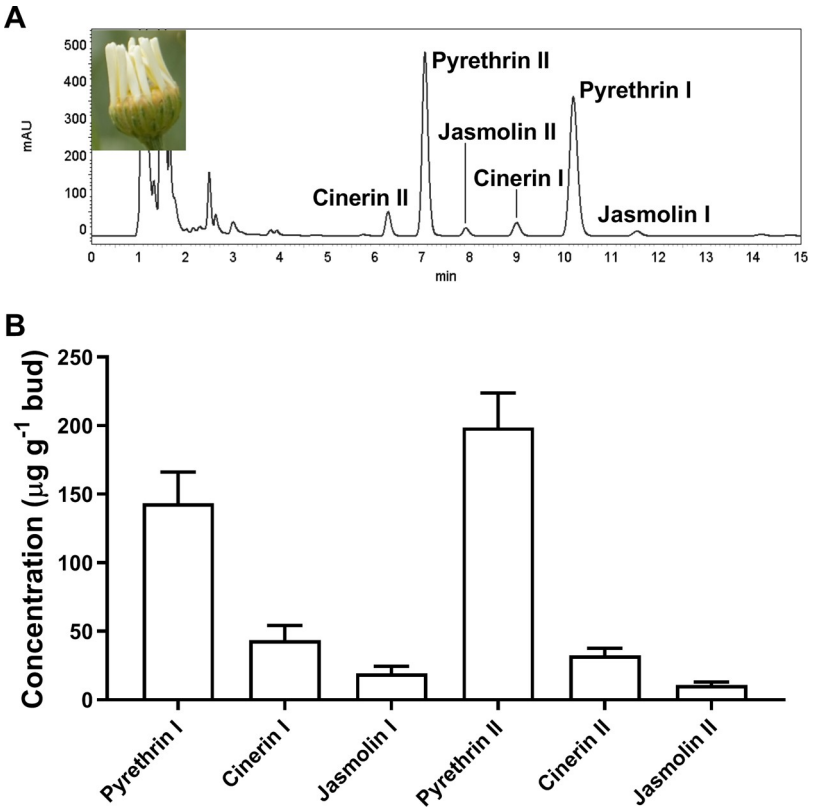

Figure 8. Pyrethrin composition in bud extracts of *T. cinerariifolium*. (**A**) HPLC profile of the acetone extract of *T. cinerariifolium* buds. (**B**) Concentrations of pyrethrin I/II, cinerin I/II, and jasmolin I/II in the bud extracts (mean ± standard error of the mean (n = 5)).

(1*R*,3*R*)-Chrysanthemic acid thioesters of *N*-acetylcysteamine (**2**) and pantetheine (**3**) showed higher affinity than chrysanthemoyl CoA (**1**) for TcGLIP (Table 1). To elucidate the mechanism, we have investigated the effects of preincubation with 10 μM CoA, *N*-acetylcysteamine, and pantetheine on the reaction of (1*R*,3*R*)-chrysanthemoyl CoA and (*S*)-pyrethrolone (**7**) at TcGLIP. The slopes of regression lines for the Lineweaver−Burk plots obtained in the presence of these inhibitors (CoA, 258.6 ± 8.0; pantetheine, 304.9 ± 9.1; *N*-acetylcysteamine, 318.3 ± 6.6) were steeper than those observed in their absence (211.3 ± 16.0, one-way ANOVA, Bonferroni test, $p < 0.05$). Also, the regression lines with and without the inhibitors intercepted the *Y*-axis at similar values with no significant difference (Figure 6), suggesting that CoA, pantetheine, and *N*-acetylcysteamine interact competitively with (1*R*,3*R*)-chrysanthemoyl CoA at TcGLIP.

We also investigated the effects of 10 μM CoA on the interactions of *S*-(1*R*,3*R*)-chrysanthemoyl pantetheine (**2**) and *S*-(1*R*,3*R*)-chrysanthemoyl *N*-acetylcysteamine (**3**) with TcGLIP. CoA appeared to increase the *Y*-axis intercept of the regression line for both compounds. Indeed, such an effect was significant for compound **2** ($p < 0.05$, one-tailed *t*-test) (Figure 6), suggesting that CoA is likely to interact noncompetitively with compound **2** at TcGLIP. If this is the case, the adenosyl moiety of CoA plays a role in such interactions.

Whatever the inhibition mechanism, no feedback inhibition by CoA has been reported to date in lipases. It is conceivable that CoA balances an acceleration of the TcGLIP reaction by the hydrophobic property of pyrethrins and the *TcGLIP* gene multiplication in the genome.[36] Also, the CoA moiety may assist in colocalizing TcGLIP and the substrates.

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In addition to the acyl CoA substrates, we also determined the kinetic parameters in the ester-forming activity of TcGLIP for three natural and two synthetic alcohols (compounds **7**–**11**, Figure 3). The catalytic efficiency V_{max}/K_m of (*S*)-pyrethrolone (**7**) was higher than that of cinerolone (**8**) and jasmololone (**9**) (Figure 7 and Table 1), pointing to the importance of the conjugated diene in the side chain in the catalytic activity. (*S*)-Allethrolone (**10**) and (*S*)-propargyllone (**11**) were inferior to the natural alcohol substrates (**7**–**9**) in terms of the catalytic efficiency (Figure 7 and Table 1), demonstrating an essential role of the side chain with four carbons or longer in the interactions with TcGLIP. In summary, of the natural rethrolones, (*S*)-pyrethrolone (**7**) showed the highest catalytic efficiency for TcGLIP, followed by (*S*)-cinerolone (**8**) and (*S*)-jasmololone (**9**). Synthetic substrates (*S*)-allethrolone (**10**) and (*S*)-propargyllone (**11**) had lower catalytic efficiency than the natural rethrolones (Figure 7 and Table 1).

To examine whether the substrate specificity of TcGLIP affects the pyrethrin composition in *T. cinerariifolium*, we quantified the six pyrethrins in the buds of the plant species. The six pyrethrins were present in the concentration order of pyrethrins I/II > cinerins I/II > jasmolins I/II in the buds of *T. cinerariifolium* (Figure 8), similar to the order of catalytic efficiency of (*S*)-pyrethrolone (**7**) > (*S*)-cinerolone (**8**) > (*S*)-jasmololone (**9**) for TcGLIP (Figure 7 and Table 1). Hence, TcGLIP, as well as the relative concentrations of the precursors, will co-contribute to any difference in the production of the pyrethrins.

In conclusion, we have found for the first time that the CoA and the side chain moieties in rethrolones are determinants of the ester-forming reaction of TcGLIP. Notably, (*S*)-pyrethrolone (**7**) was more favorable than (*S*)-cinerolone (**8**) and (*S*)-jasmololone (**9**) for the interactions with TcGLIP, explaining, at least in part, for the higher production of pyrethrin I/II than cinerin I/II and jasmolin I/II.[1,2,37] Therefore, modification of TcGLIP in the catalytic and allosteric sites can enhance pyrethrin production, change the six pyrethrin compositions, and help control pest insect species with these organic insecticides.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.2c02365.

HPLC data of all of the tested compounds are shown in Figures S1 and S2; analytical data of compounds (**2**)–(**6**) are summarized in Tables S1–S5 and their NMR spectra are shown in Figures S3–S7, and the concentration-specific activity relationship of compound (**6**) for TcGLIP is illustrated in Figure S8 (PDF)

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Author Contributions

Y.S., S.A., K.K., and M.I. prepared substrates and investigated the enzyme kinetics. Y.S. and M.I. quantified pyrethrins in *T. cinerariifolium*. Y.S., M.I., and K.M. analyzed data and wrote the paper.

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Notes

The authors declare no competing financial interest.

All data used in this study are available upon request.

Approval for experiments with genetically modified microorganisms

All the experiments with genetically modified microorganisms were approved by Kindai University (Number KDAS-16-016).

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■ ABBREVIATIONS

CoA, coenzyme A; DTT, dithiothreitol; GELP, GDSL esterase/lipase protein; TcGLIP, *Tanacetum cinerariifolium* GDSL lipase; THF, tetrahydrofuran

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