

Characterization of steroid 5 α -reductase involved in α -tomatine biosynthesis in tomatoes

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Received September 26, 2019; accepted October 30, 2019 (Edited by T. Koezuka)

Abstract α -tomatine and dehydrotomatine are steroidal glycoalkaloids (SGAs) that accumulate in the mature green fruits, leaves, and flowers of tomatoes (*Solanum lycopersicum*) and function as defensive compounds against pathogens and predators. The aglycones of α -tomatine and dehydrotomatine are tomatidine and dehydrotomatidine (5,6-dehydrogenated tomatidine), and tomatidine is derived from dehydrotomatidine via four reaction steps: C3 oxidation, isomerization, C5 α reduction, and C3 reduction. Our previous studies (Lee et al. 2019) revealed that *Sl3 β HSD* is involved in the three reactions except for C5 α reduction, and in the present study, we aimed to elucidate the gene responsible for the C5 α reduction step in the conversion of dehydrotomatidine to tomatidine. We characterized the two genes, *SIS5 α R1* and *SIS5 α R2*, which show high homology with *DET2*, a brassinosteroid 5 α reductase of *Arabidopsis thaliana*. The expression pattern of *SIS5 α R2* is similar to those of SGA biosynthetic genes, while *SIS5 α R1* is ubiquitously expressed, suggesting the involvement of *SIS5 α R2* in SGA biosynthesis. Biochemical analysis of the recombinant proteins revealed that both of *SIS5 α R1* and *SIS5 α R2* catalyze the reduction of tomatid-4-en-3-one at C5 α to yield tomatid-3-one. Then, *SIS5 α R1*- or *SIS5 α R2*-knockout hairy roots were constructed using CRISPR/Cas9 mediated genome editing. In the *SIS5 α R2*-knockout hairy roots, the α -tomatine level was significantly decreased and dehydrotomatine was accumulated. On the other hand, no change in the amount of α -tomatine was observed in the *SIS5 α R1*-knockout hairy root. These results indicate that *SIS5 α R2* is responsible for the C5 α reduction in α -tomatine biosynthesis and that *SIS5 α R1* does not significantly contribute to α -tomatine biosynthesis.

Key words: α -tomatine, CRISPR/Cas9, hairy roots, *Solanum lycopersicum*, steroidal glycoalkaloid.

Introduction

Steroidal glycoalkaloids (SGAs) are typically found in members of *Solanum* species, and are known as toxic substances in *Solanum* food crops (Harrison 1990; Helmut 1998; Petersen et al. 1993) such as tomato (*Solanum lycopersicum*), potato (*Solanum tuberosum*), and eggplant (*Solanum melongena*). Because of their toxic effects on fungi, bacteria, insects, and animals, SGAs are considered to play defensive roles against a wide range of pathogens and predators (Friedman 2002, 2006). Tomato contains α -tomatine and dehydrotomatine as major SGAs in green tissues such as leaves and

immature fruits (Friedman 2002). However, during the fruits ripening of tomato fruits, α -tomatine accumulated in immature fruits is metabolized and converted to nontoxic SGA esculeoside A (Iijima et al. 2009). In potatoes, α -solanine and α -chaconine are the two major SGAs, and their contents are especially high in tuber sprouts (Friedman 2006). Eggplant mainly produces α -solasonine and α -solamargine (Wu et al. 2013). The enormous structural diversity of SGAs is generated by various combinations of steroidal aglycones and sugar residues.

The common precursor for the biosynthesis of SGAs is cholesterol (Sawai et al. 2014), which undergoes

Abbreviations: SGA, steroidal glycoalkaloid; CYP, cytochrome P450 monooxygenase; DOX, 2-oxoglutarate dependent dioxygenase; S5 α R, steroid 5 α -reductase; UGT, UDP-dependent glycosyltransferase; 3 β HSD, 3 β -hydroxysteroid dehydrogenase; 3KSI, 3-ketosteroid isomerase; 3KSR, 3-ketosteroid reductase.

This article can be found at <http://www.jspcmb.jp/>

Published online December 13, 2019

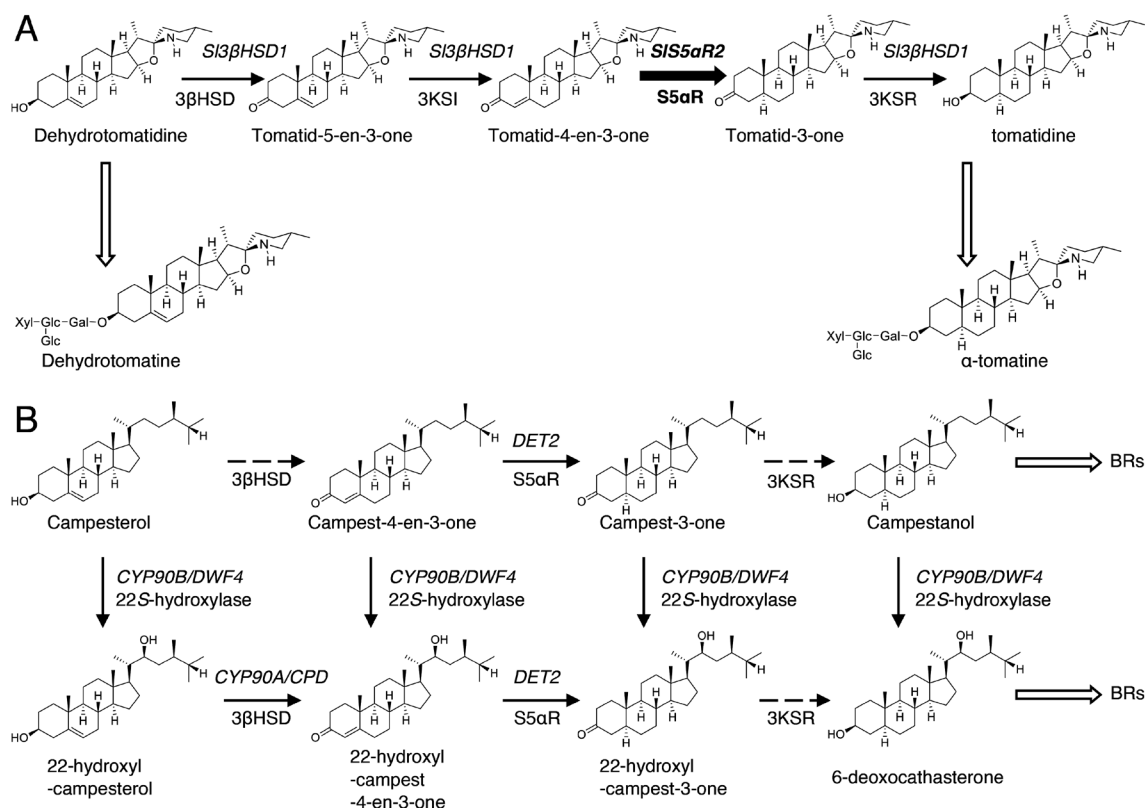


Figure 1. The putative biosynthetic pathways for (A) α -tomatine in tomatoes and (B) brassinosteroids (BRs) in plants. The thick solid arrow indicates the reaction step characterized in this work. Black thin solid arrows indicate the single reaction step characterized previously, and dashed arrows indicate the uncharacterized step. White thick arrows represent multiple reaction steps. The names of enzymes are indicated under the arrows. $3\beta\text{HSD}$, 3β -hydroxysteroid dehydrogenase; 3KSI , 3-ketosteroid isomerase; $S5\alpha\text{R}$, steroid 5α -reductase; 3KSR , 3-ketosteroid reductase. The genes previously characterized are shown in the upper part of the arrows. $Sl3\beta\text{HSD1}$ involved in the conversion of dehydropomatidine to tomatidine in tomatoes; $DWF4$, *dwarf4*; $DET2$, *de-etiolated-2*; CPD , *constitutive photomorphogenesis dwarf*.

subsequent modification via oxidation at the C16, C22, and C26 positions, transamination at the C26, and glycosylation at the C3 hydroxy group (Friedman 2002; Ginzberg et al. 2009; Ohyama et al. 2013; Petersen et al. 1993). Recently, several SGA biosynthetic genes were identified in tomatoes and potatoes. For instance, sterol side chain reductase 2 (SSR2) was identified as a key enzyme in cholesterol and the resulting SGAs biosynthesis (Sawai et al. 2014), and two cytochrome P450 monooxygenases (CYPs) (PGA1/CYP72A208 and PGA2/CYP72A188) and a 2-oxoglutarate-dependent dioxygenase (DOX) (16DOX) are responsible for hydroxylation of cholesterol at the C26, C22 and C16 positions, respectively, to produce SGAs (Itkin et al. 2013; Nakayasu et al. 2017; Umemoto et al. 2016). Additionally, the involvement of some UDP-dependent glycosyltransferases (UGTs), including GAME1 in tomato, were found to be involved in glycosylation steps in biosynthesis of SGAs (Itkin et al. 2011). However, the genes involved in the later biosynthetic processes have not yet been elucidated.

As described above, tomato contains α -tomatine and dehydropomatine as major SGAs (Friedman 2002). α -Tomatine and dehydropomatine are biosynthesized

via four steps of glycosylations from their respective aglycones, tomatidine and dehydropomatidine. Tomatidine possesses a single bond between C5 and C6 (dihydro type), while dehydropomatidine has a $\Delta^{5,6}$ double bond (dehydro type), and is biosynthesized from dehydropomatidine by the four-step reaction process shown in Figure 1. We recently identified that the gene designated as $Sl3\beta\text{HSD1}$ is involved in the conversion of dehydropomatidine to tomatidine (Lee et al. 2019). $Sl3\beta\text{HSD1}$ is a multifunctional enzyme that possesses the activities of 3β -hydroxysteroid dehydrogenase ($3\beta\text{HSD}$) and 3-ketosteroid isomerase (3KSI) when acting on dehydropomatidine to form tomatid-4-en-3-one and also shows the activity of 3-ketosteroid reductase (3KSR) when acting on tomatid-3-one to produce tomatidine (Lee et al. 2019). Therefore, $Sl3\beta\text{HSD1}$ is involved in the three reaction steps among the four reactions from dehydropomatidine to tomatidine, and however, it has not yet been identified as the gene involved in a 5α -reduction step from tomatid-4-en-3-one to tomatid-3-one.

The 5α -reduction step is thought to be catalyzed by a steroid 5α -reductase ($S5\alpha\text{R}$), which is involved in the NADPH-dependent reduction of the $\Delta^{4,5}$ double bond in various steroids and steroid hormones. In plants,

Arabidopsis *DET2* (*AtDET2*) has been identified as a biosynthetic gene of plant hormone brassinosteroids, and it encodes an 5α R protein that shares significant sequence identity with mammalian 5α Rs (Noguchi et al. 1999). *AtDET2* catalyzes the NADPH-dependent reduction of the $\Delta^{4,5}$ double bond in various steroids. The Arabidopsis *det2* mutant shows a small dark-green dwarf phenotype, as a consequence of brassinosteroid deficiency via lack of the conversion of campest-4-en-3-one to campest-3-one (Noguchi et al. 1999). Tomato *LeDET2*, which is an ortholog of *AtDET2* has been characterized as a functional steroid 5α -reductase (Rosati et al. 2005). Moreover, the presence of two isozymes was suggested by a crude enzyme assay performed on tomatoes (Rosati et al. 2005). We recently isolated and characterized *SIS5 α R1*, and co-incubation of *SIS5 α R1* with *Sl3 β HSD1* and dehydrotomatidine in vitro demonstrated that the four reaction steps converting dehydrotomatidine to tomatidine were reconstituted in the in vitro assay (Lee et al. 2019). However, whether *SIS5 α R1* contributes to α -tomatine biosynthesis in vivo has remained unknown.

In the present study, we isolated a second *DET2* homolog from tomato, designated as *SIS5 α R2*. The expression pattern of *SIS5 α R2* was similar to those of known SGA biosynthetic genes in tomato. In vitro assay of recombinant *SIS5 α R2* using progesterone as a substrate revealed that *SIS5 α R2* encodes a functional steroid 5α -reductase. Additionally, simultaneous incubation of *SIS5 α R2* with *Sl3 β HSD1* resulted in the successive conversion of dehydrotomatidine to tomatidine in vitro, as observed in the case of recombinant *SIS5 α R1*. Furthermore, CRISPR/Cas9-mediated knockout of either *SIS5 α R2* or *SIS5 α R1* was conducted in tomato hairy roots. While disruption of *SIS5 α R1* did not affect the endogenous SGA levels, *SIS5 α R2*-knockout tomato hairy roots showed drastic reduction in the α -tomatine level and significant accumulation of dehydrotomatine. Based on all these findings, we conclude that *SIS5 α R2*, but not *SIS5 α R1*, is essential for the 5α -reduction step of α -tomatine biosynthesis in tomatoes.

Materials and methods

Plant materials

The tomato plant used in this study was *S. lycopersicum* cv Micro-Tom (TOMJPF00001), obtained from the NBRP (MEXT, Japan). The seeds were germinated and cultivated under a 16/8-h light/dark photoperiod in plant thermostatic chambers at 25°C until the plants produced ripening red fruits.

Chemicals

Progesterone were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), and 5α -pregnane-3,20-dione was purchased from Santa Cruz Biotechnology (Santa Cruz,

CA, USA). Dehydrotomatidine was isolated in our lab from tomatidine purchased from Chromadex (Irvine, CA, USA) using high-performance liquid chromatography (HPLC). α -Tomatine was purchased from Sigma-Aldrich.

RNA extraction and reverse transcription

Total RNAs were extracted using the RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) and the RNase-Free DNase Set (QIAGEN) according to the manufacturer's instructions. The extracted total RNAs were used to synthesize the first strand of cDNA using the Transcriptor First Strand cDNA Synthesis Kit (TOYOBO, Osaka, Japan) for real-time quantitative polymerase chain reaction (PCR).

Real-time quantitative PCR analysis

Real-time quantitative PCR (qPCR) was performed with the LightCycler[®]Nano (Roche, Basel, Switzerland) using GeneAmp SYBR[®] qPCR Mix α (Nippon Gene), with the following primers 1 and 2 for *SIS5 α R1*, 3 and 4 for *SIS5 α R2*, 5 and 6 for *ubiquitin* (Supplementary Table S2), using as template cDNAs from the various tomato tissues; flower, green fruit (G fruit), yellow fruit (Y fruit), orange fruit (O fruit), and red fruit (R fruit) as templates. Cycling was conducted at 95°C for 10 min, 45 cycles at 95°C for 30 s, and 60°C for 1 min for amplification, followed by holding at 95°C for 30 s and ramping up from 60 to 95°C at 0.1°C s⁻¹ to perform a melting curve analysis. Each assay was repeated three times. The expression levels were normalized against the values obtained for the *ubiquitin* gene, which was used as an internal reference in tomato. Data acquisition and analysis were carried out using LightCycler[®]Nano software (Roche).

Cloning of *SIS5 α R1*, *SIS5 α R2* and *Sl3 β HSD1*

The DNA fragments containing the ORF of *Solyc09g013070* (*SIS5 α R1*) and *Solyc10g086500* (*SIS5 α R2*) were isolated using the cDNA template from the tomato leaves with primer sets 7 and 8 for *SIS5 α R1*, and 9 and 10 for *SIS5 α R2* (Supplementary Table S2). The PCR product was purified using Wizard[®] SV Gel and the PCR Clean-Up System (Promega, WI, USA) and cloned into the pMD19 T-vector (TaKaRa, Shiga, Japan). The sequence of the cloned DNA was confirmed using the ABI 3130 genetic analyzer (Applied Biosystems, CA, USA) and analyzed using BioEdit, a biological sequence alignment editor (<http://www.mbio.ncsu.edu/>). Similarly, the open reading frame (ORF) of *Sl3 β HSD1* was isolated using a clone of the LEFL1039AB03 (identical to *Solyc01g073640* in the tomato ITAG 2.4) in the Kazusa Full-length Tomato cDNA Database (<http://www.pgb.kazusa.or.jp/kaftom/clone.html>), purchased from Kazusa DNA Research Institute. The ORF of *Sl3 β HSD1* was amplified using PCR with the clone as a template and primer sets 11 and 12 (Supplementary Table S2).

Heterologous expression of the recombinant *SIS5 α R1*, *SIS5 α R2* and *Sl3 β HSD1* proteins

The DNA fragments containing the ORF of the *SIS5 α R1* and

SIS5 α R2 gene were ligated into a pFastBac1 vector (Invitrogen, Carlsbad, CA), and were then introduced into *Escherichia coli* DH10Bac (Life Technologies) to generate the corresponding recombinant bacmid DNAs. Preparation of recombinant baculovirus DNAs that contained *SIS5 α R1* and *SIS5 α R2* ORFs and transfection of *Spodoptera frugiperda* 9 insect cells were carried out according to the manufacturer's instructions (Life Technologies). Heterologous expression of *SIS5 α R1* and *SIS5 α R2* proteins in insect cells was conducted as described by Ohnishi et al. (2006). Similarly, the DNA fragments containing the *Sl3 β HSD1* gene ORF were ligated into pCold ProS2 vector (TaKaRa), and recombinant *Sl3 β HSD1* protein was prepared using bacterial expression system in *E.coli* strain BL21 (DE3) as described by Lee et al. (2019).

***In vitro* enzyme activity assay**

To prepare tomatid-4-en-3-one, we conducted in vitro enzyme assay of recombinant *Sl3 β HSD1* protein, and the assay was performed using 100 μ l of reaction mixture comprising 100 mM bis-tris-HCl (pH 7.2), 2.5 mM NAD⁺ as a coenzyme, 50 μ M dehydrotomatidine as a substrate, and the purified *Sl3 β HSD1*. The enzymatically synthesized tomatid-4-en-3-one was further metabolized by recombinant *SIS5 α R1* to produce tomatid-3-one, and the reaction was carried out as described below.

Microsomal fractions of the insect cells expressing *SIS5 α R1* or *SIS5 α R2* were obtained from 100 ml of cultured cell suspension. Infected cells were washed with phosphate-buffered saline and suspended in buffer A, consisting of 20 mM potassium phosphate (pH 7.5), 20% (v/v) glycerol, 1 mM ethylenediaminetetraacetic acid, and 1 mM dithiothreitol. The cells were sonicated and cell debris was removed by centrifugation at 10,000 *g* for 15 min, and the resulting pellets were homogenized with buffer A to provide the microsomal fractions. The assay of *SIS5 α R1* and *SIS5 α R2* for steroid C5 reduction were performed using 100 μ l reaction mixture comprising 100 mM potassium phosphate (pH 7.2), 1 mM NADPH or NADH as a coenzyme, the enzymatically synthesized tomatid-4-en-3-one, and the microsomal fraction containing *SIS5 α R1* or *SIS5 α R2*. The conversion of dehydrotomatidine to tomatidine was reconstituted in an in vitro assay by mixing the purified *Sl3 β HSD1* protein with *SIS5 α R1* or *SIS5 α R2*-containing microsomes. The reaction mixture consisted of 100 mM bis-tris-HCl buffer (pH 7.2), purified *Sl3 β HSD1* protein, microsomes containing *SIS5 α R1* or *SIS5 α R2*, 2.5 mM NAD⁺, 1 mM NADPH, and 25 μ M dehydrotomatidine. All the reactions were performed at 30°C for 2 h and were terminated by the addition of 100 μ l ethyl acetate. The reaction products were extracted three times with an equal volume of ethyl acetate and the organic phase was collected and evaporated. The residues were analyzed using gas chromatography-mass spectrometry (GC-MS) and/or liquid chromatography-mass spectrometry (LC-MS).

GC-MS analysis of the reaction product was conducted as described previously (Lee et al. 2019). Briefly, the residue was trimethylsilylated, and then subjected to a GC-MS-

QP2010 Ultra (Shimadzu, Kyoto, Japan) with a DB-1MS (30 m \times 0.25 mm, 0.25 μ m film thickness; J&W Scientific, CA, USA) capillary column. An MS scan mode with a mass range of *m/z* 50–700 were used. Progesterone and 5 α -pregnane-3,20-dione were monitored at a selected ion monitoring (SIM) mode chromatogram at *m/z* 229 and 231, respectively. Tomatid-4-en-3-one and tomatid-3-one were analyzed via SIM mode at *m/z* 125. For LC-MS analysis, the residue was dissolved in 200 μ l of methanol. LC-MS analysis of each reaction product was carried out as described above Lee et al. (2019), using an ACQUITY UPLC H-Class System (Waters, MA, USA) with an Acquity UPLC HSS-T3 column (1.8 μ m, 2.1 \times 100 mm², Waters) at 40°C and an SQ Detector 2 (Waters). Data acquisition and analysis were performed using MassLynx 4.1 software (Waters). Reaction products were analyzed by SIM-mode chromatography at *m/z* 412, 414, and 416.

Construction of CRISPR/Cas9 vectors

The knockout tomato hairy roots for each of *SIS5 α R1* and *SIS5 α R2* were generated by targeted genome editing with the CRISPR/Cas9 system. We used the CRISPR/Cas9 binary vector pMgP237-2A-GFP to express multiplex gRNAs (Hashimoto et al. 2018; Nakayasu et al. 2018). To design a gRNA target with low off-target effect in *SIS5 α R1* and *SIS5 α R2*, we conducted in silico analyses using the Web tool Design sgRNAs for CRISPRko (<https://portals.broadinstitute.org/gpp/public/analysis-tools/sgRNA-design>) and Cas-OT software (Xiao et al. 2014). Then we selected target sequences named *SIS5R1_492-511* and *SIS5R1_552-571* in the coding region of *SIS5 α R1*, and *SIS5R2_449-468* and *SIS5R2_511-530* in the coding region of *SIS5 α R2*, respectively. To enhance the efficiency of gRNA transcription from U6 promoter, one G was added to the 5'-end of *SIS5R1_492-511*, *SIS5R1_552-571* and *SIS5R2_511-530*. Two DNA fragments composed of the gRNA scaffold and tRNA scaffold between two target sequences, *SIS5R1_492-511/SIS5R1_552-571* and *SIS5R2_449-468/SIS5R2_511-530*, were generated by PCR using pMD-gRNA containing gRNA and tRNA scaffolds as a template and primer sets containing restriction enzyme *BsaI* sites (13 and 14 for *SIS5R1_492-511/SIS5R1_552-571*, 15 and 16 for *SIS5R2_449-468/SIS5R2_511-530*) (Supplementary Table S2). The units containing two gRNAs-tRNAs were then independently inserted into the *BsaI* site of pMgP237-2A-GFP using Golden Gate Cloning methods to construct the CRISPR/Cas9 vectors, labeled pMgP237-*SIS5 α R1ko* and pMgP237-*SIS5 α R2ko*, respectively.

Generation of SIS5 α R1- and SIS5 α R2-disrupted transgenic tomato hairy roots

The vectors pMgP237-*SIS5 α R1ko* and pMgP237-*SIS5 α R2ko* were independently introduced into *Agrobacterium rhizogenes* ATC15834 by electroporation. Transgenic tomato hairy roots were generated as previously described by Thagun et al. (2016). Genome DNA was extracted from each established hairy root and transformants were selected by genomic PCR

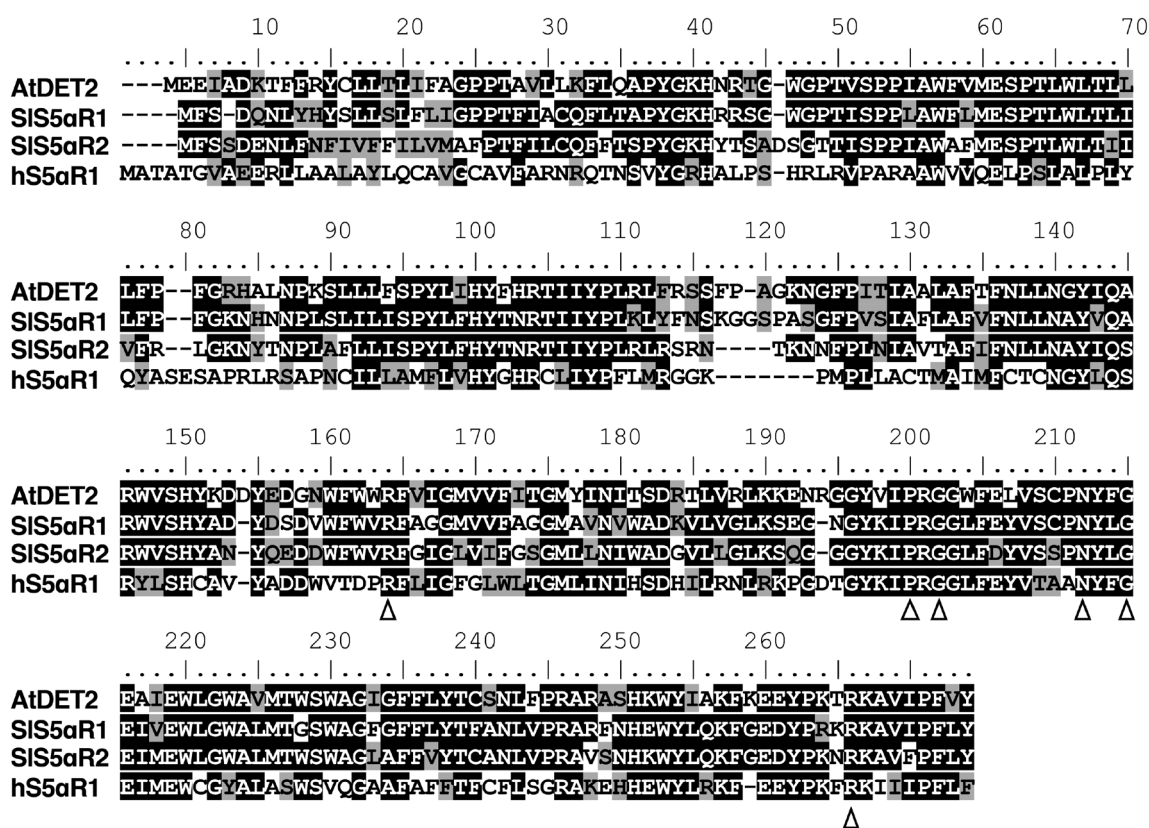


Figure 2. Multiple sequence alignment of SIS5αR1 and SIS5αR2 with AtDET2 from *Arabidopsis thaliana* and human hS5αR1. Black and gray shades indicate identical and similar amino acid residues, respectively. Conserved residues, which are required for steroid 5α-reduction activity, are marked by white triangles.

to amplify a partial fragment of the T-DNA region integrated into the genome using primers 17 and 18 (Supplementary Table S2). To analyze the mutations or large deletions in the transgenic hairy roots, the region including the target sites of gRNAs was amplified by PCR using primers 19 and 20 for pMgP237-SIS5αR1ko transformants and primers 21 and 22 for pMgP237-SIS5αR2ko transformants (Supplementary Table S2). The PCR fragments obtained from each transformant were then subjected to agarose-gel electrophoresis or microchip electrophoresis using MultiNA (Shimadzu). Furthermore, each PCR fragment from four independent transgenic hairy root lines (pMgP237-SIS5αR1ko_#1 and #6, pMgP237-SIS5αR2ko_#7 and #12) was cleaned using the Wizard[®] SV Gel and PCR Clean-Up System (Promega, Japan) according to the manufacturer's instructions, and cloned into pCR[™]4Blunt-TOPO[®] (Invitrogen). Sanger sequencing of each of the cloned DNAs was performed using a sequencing service (Eurofins Genomics).

Quantification of SGAs in tomato hairy roots

SGAs were extracted from 100 mg fresh weight of harvested transgenic lines, and each sample was prepared and analyzed by LC-MS. The LC-MS analysis was performed using the LC-MS/MS system; water with 0.1% (v/v) formic acid (A) and acetonitrile (B) were used as mobile phases, with an elution gradient of 10–55% B from 0 to 30 min and 55–75% B from

30 to 35 min (linear gradient). MS scan mode was used with a mass range of m/z 400–1,200. The quantities of α-tomatine and dehydrotomatine were calculated from the peak area using an α-tomatine calibration curve.

Phylogenetic analysis

Amino acid sequences of AtDET2 homologs were retrieved from the Phytozome (<https://phytozome.jgi.doe.gov/pz/portal.html>) and Sol Genomics networks (<https://solgenomics.net/>). A phylogenetic tree was constructed using the MEGA7 program and the maximum likelihood method with the following parameters: Poisson correction, pairwise deletion and bootstrap (1,000 replications; random seed).

Results

Selection of candidate S5αR genes involved in α-tomatine biosynthesis

To survey the candidate gene encoding functional S5αR, BLASTX analysis against tomato transcripts database from Sol Genomics (<http://solgenomics.nrt/>) used AtDET2 as a query. This analysis allowed us to select two distinct cDNAs encoded by *Solyc09g013070* and *Solyc10g086500*. *Solyc09g013070* was identical to *SIS5αR1*, which has been already characterized to encode a functional steroid 5α-reductase (Lee et al. 2019), and

Solyc10g086500 was identical to *LeDET2*, which was previously characterized to encode a functional steroid 5 α -reductase (Rosati et al. 2005), and here we designated it as *SIS5 α R2*. *SIS5 α R1* and *SIS5 α R2* show 63% and 57% amino acid identity to *AtDET2*, respectively, and share 67% amino acid identity to each other. Both *SIS5 α R1* and *SIS5 α R2* contain six (Arg-150/148, Pro-185/183, Gly-187/185, Asn-197/195, Gly-200/198 and Arg-251/249) of seven conserved amino acid residues that are part of a typical cofactor binding domain in mammalian 5 α -reductase (Figure 2; Russell and Wilson 1994). Both *SIS5 α R1* and *SIS5 α R2* contain six potential transmembrane-spanning domains based on the TMHMM program (<http://www.cbs.dtu.dk/services/TMHMM>), as well as *AtDET2* predicted by the same program.

Expression analysis of *SIS5 α R1* and *SIS5 α R2* in various tissues of tomato

The expression of *SIS5 α R1* and *SIS5 α R2* was analyzed in the RNA-seq data of the Tomato Functional Genomics Database (<http://ted.bti.cornell.edu/>). *SIS5 α R2* showed an expression pattern similar to those of the known SGA biosynthetic genes in terms of higher expression in immature fruits and decreasing expression levels during ripening of fruits, whereas the transcript levels of *SIS5 α R1* remained high even in red ripe fruits where the expression of SGA genes was low (Supplementary Table S1). To confirm the expression of *SIS5 α R1* and *SIS5 α R2*, real-time PCR was performed using the reverse-transcription products of total RNAs extracted from various tissues of *S. lycopersicum* cv. Micro-Tom. *SIS5 α R2* was highly expressed in flowers, where a large amount of α -tomatine is accumulated (Roddick 1974), while the expression level of *SIS5 α R2* was low in red mature fruits (Figure 3), in which α -tomatine is further metabolized to esculocidine A and the α -tomatine level is very low (Iijima et al. 2009). This expression pattern of *SIS5 α R2* is similar to those of the SGA biosynthetic genes *Sl16DOX*, *GAME1*, and *Sl3 β HSD1* (Lee et al. 2019; Nakayasu et al. 2017). On the other hand, *SIS5 α R1* was abundantly expressed in red mature fruits, and the expression pattern of *SIS5 α R1* is not in agreement with the known SGA biosynthetic genes (Figure 3).

In vitro functional analysis of the C5 α -reduction activity of *SIS5 α R2* on tomatid-4-en-3-one to tomatid-3-one

In our recent studies, the recombinant *SIS5 α R1* protein was expressed with a baculovirus-mediated expression system in *Spodoptera frugiperda* 9 insect cells and confirmed to catalyze C5 α -reduction of tomatid-4-en-3-one to yield tomatid-3-one in vitro (Lee et al. 2019). Previously, it was reported that recombinant *SIS5 α R2* expressed in mammalian cells (COS-7) was active on

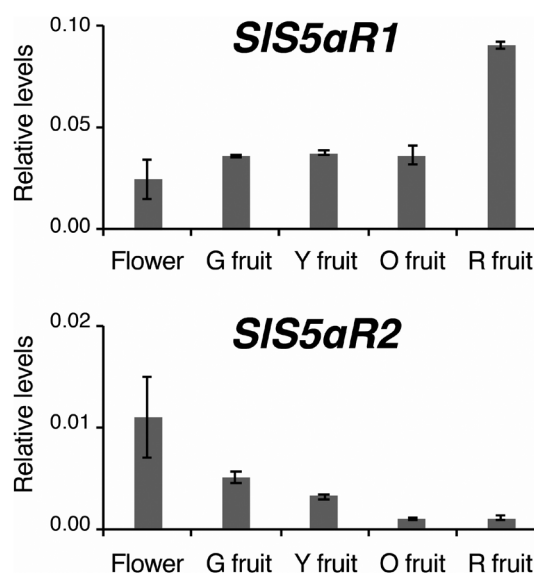


Figure 3. Quantitative RT-PCR analysis of *SIS5 α R1* and *SIS5 α R2* expression in various tissues of tomatoes. G, Y, O, and R mean green, yellow, orange, and red, respectively. Transcript levels of *SIS5 α R1* and *SIS5 α R2* are shown relative to that of the *ubiquitin* gene as an internal reference. Error bars indicate standard error from the mean ($n=3$).

typical substrates of mammalian S5 α Rs (progesterone, testosterone and androstenedione) (Rosati et al. 2005). In the present study, to compare the catalytic activities of *SIS5 α R1* and *SIS5 α R2*, the recombinant *SIS5 α R2* was prepared with a baculovirus-mediated expression system and C5-reduction activities were analyzed. First, the functional expression of *SIS5 α R2* was confirmed by in vitro enzyme assays using progesterone as a substrate in the presence of NADPH or NADH as a coenzyme, and the reaction products were analyzed by gas chromatography-mass spectrometry (GC-MS). A product with a retention time of 9.2 min and a major mass fragment ion at m/z 231 was detected. This product was identical to the authentic compound 5 α -pregnane-3,20-dione in terms of both the retention time the mass spectrum (Figure 4). Although *SIS5 α R2* could catalyze the C5 reduction with either NADPH or NADH as a coenzyme, the reduction activity in the presence of NADPH was much higher than that in the presence of NADH. Thus, the functional expression of *SIS5 α R2* in the baculovirus-insect cell system was confirmed.

We evaluated the C5 α -reduction activities on tomatid-4-en-3-one. Because authentic compounds of tomatid-4-en-3-one and tomatid-3-one are not commercially available, we obtained them by enzymatic synthesis using *Sl3 β HSD1* and *SIS5 α R1* (Lee et al. 2019). Tomatid-4-en-3-one was synthesized from dehydrotomatidine by enzymatic conversion with the recombinant *Sl3 β HSD1* using NAD⁺ as a coenzyme, and tomatid-3-one was synthesized from tomatid-4-en-3-one by enzymatic conversion with *SIS5 α R1* using NADPH as a coenzyme.

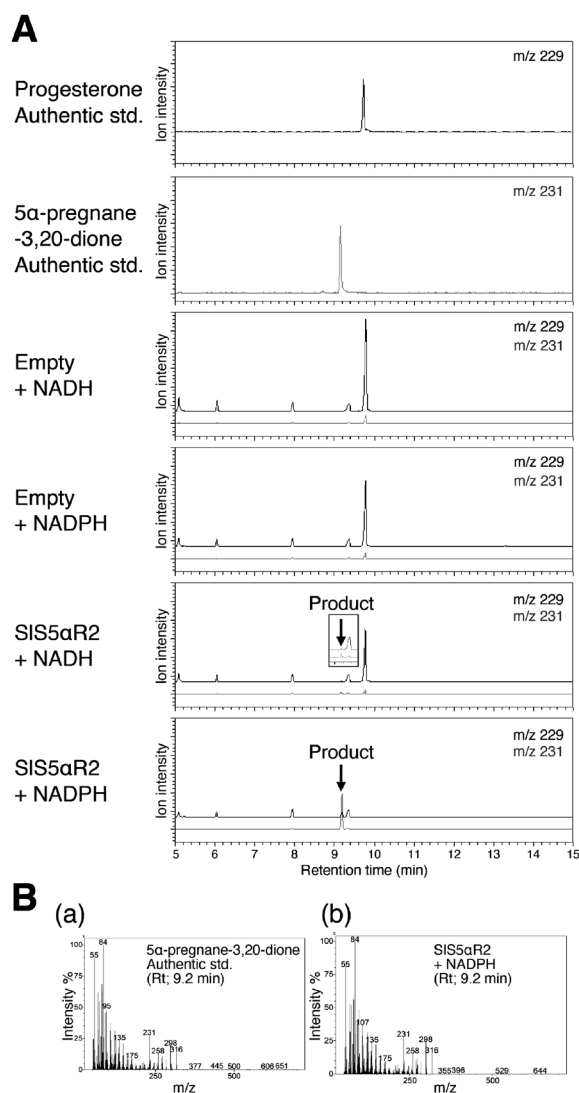


Figure 4. In vitro assay of the C5 reduction activity of SIS5 α R1 with progesterone as the substrate. (A) Progesterone was monitored by selected ion monitoring (SIM) at m/z 229 and is shown in black, and the product 5 α -pregnane-3,20-dione was monitored at m/z 231 and is shown in gray. (B) Mass spectrum of the (a) authentic 5 α -pregnane-3,20-dione at a retention time of 9.2 min and (b) product peak of progesterone incubated with SIS5 α R2 and NADPH.

Then, the recombinant SIS5 α R2 was assayed with the enzymatically synthesized tomatid-4-en-3-one in the presence of NADPH, and the reaction products were analyzed using GC-MS (Figure 5A). Tomatid-4-en-3-one was metabolized to yield a product with a retention time of 18.9 min, and this product by SIS5 α R2 was identical to tomatid-3-one, obtained by incubating tomatid-4-en-3-one with SIS5 α R1 (Figure 5A). Thus, SIS5 α R2 could catalyze the reduction of tomatid-4-en-3-one at C5 α to yield tomatid-3-one.

Next, we conducted a co-incubation assay of SIS5 α R2 with Sl3 β HSD1 using dehydrotomatidine as a substrate, and the reaction products were analyzed by LC-MS (Figure 5B). When incubating dehydrotomatidine

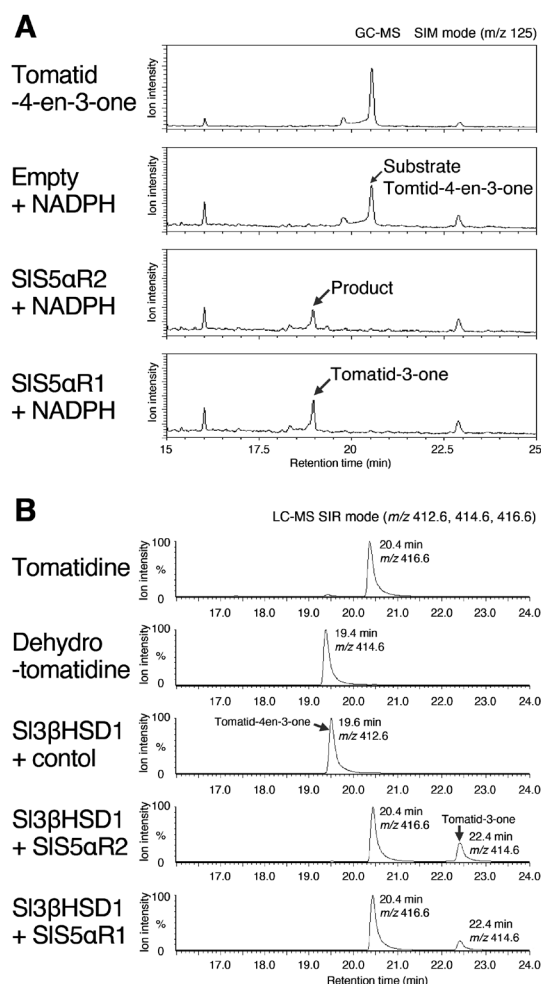


Figure 5. In vitro assays of SIS5 α R1 and SIS5 α R2 with tomatid-4-en-3-one. (A) GC-MS analysis of the in vitro assays of SIS5 α R1/2 and NADPH incubated with tomatid-4-en-3-one as a substrate. Substrate and products were monitored by SIM mode at m/z 125. (B) LC-MS analysis of the in vitro assays of SIS5 α R1/2 and Sl3 β HSD1 incubated with dehydrotomatidine as a substrate. Substrate and products were monitored by SIM mode at m/z 412, 414, and 416.

with Sl3 β HSD1, tomatid-4-en-3-one was detected with a retention time of 19.6 min and with a parent mass at m/z 412.6 (Figure 5B). Simultaneous incubation of dehydrotomatidine with SIS5 α R2 and Sl3 β HSD1 resulted in the detection of two major products with the retention times of 20.4 and 22.4 min, respectively (Figure 5B). The product with a retention time of 22.4 min gave a parent mass at m/z 414.6, which corresponded to [tomatid-3-one+H⁺]⁺ (Figure 5B). The product at a retention time of 20.4 min, which had a parent mass at m/z 416.6, was identical to the authentic tomatidine (Figure 5B). These results indicate that, during co-incubation with SIS5 α R2 and Sl3 β HSD1, dehydrotomatidine was at first converted to tomatid-4-en-3-one by Sl3 β HSD1, and SIS5 α R2 subsequently catalyzed the reduction of tomatid-4-en-3-one at C5 α to form tomatid-3-one, and Sl3 β HSD1 finally catalyzed the reduction of tomatid-3-one at C3 to

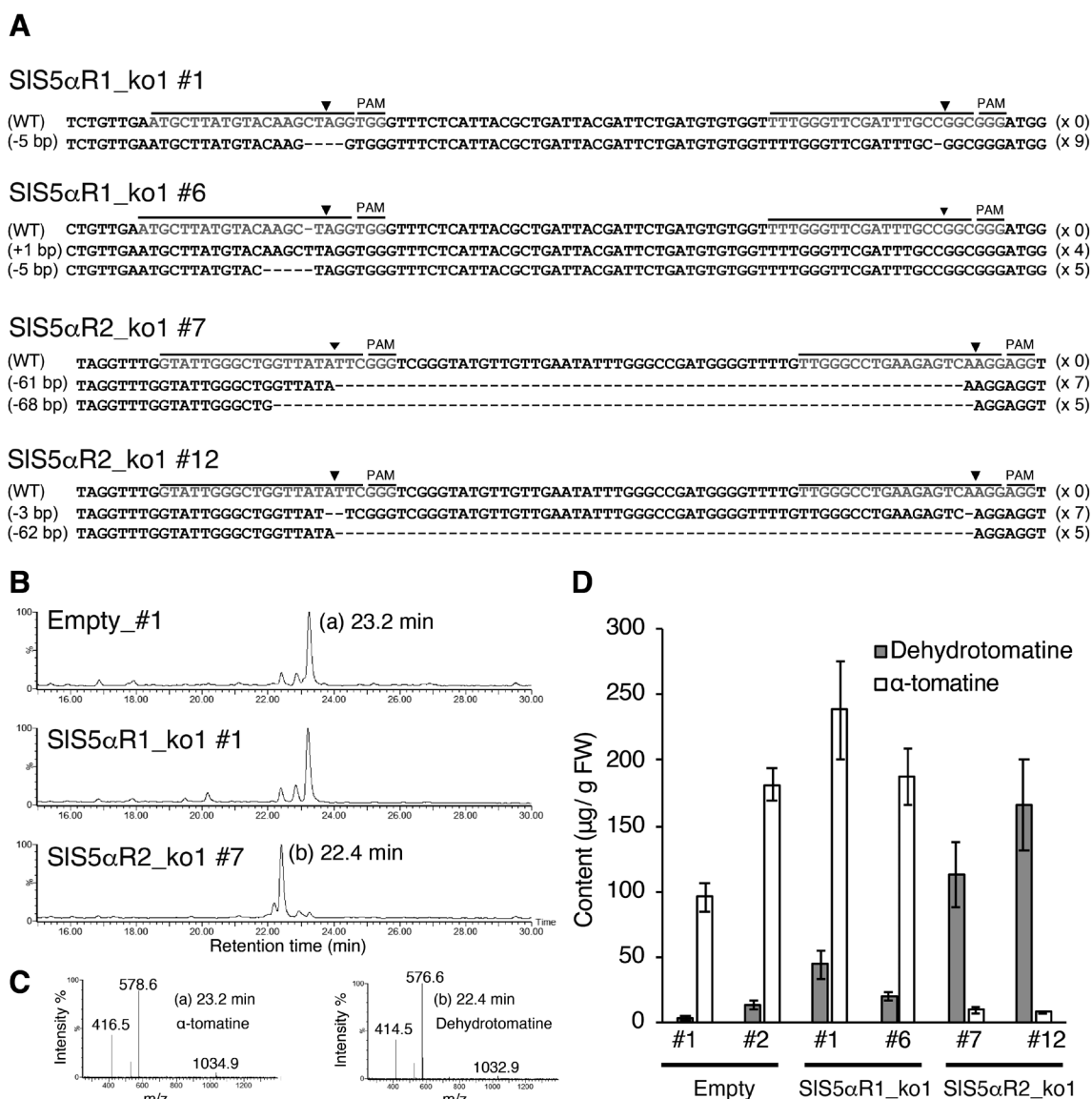


Figure 6. Characterization of *SIS5 α R1* or *SIS5 α R2*-disrupted tomato hairy roots. (A) DNA sequences of gRNA target sites. Black and gray lines indicate gRNA target sites and PAM sequences, respectively. Black triangles indicate cleavage positions of target sites. Deletions or insertions of nucleotides are presented on the left sides of the sequences, and the numbers of detected sequences are shown on the left sides. (B) LC-MS analysis of SGAs in *SIS5 α R1_ko1*_#1 and *SIS5 α R2_ko1*_#7. (C) Mass spectrum of the α -tomatine (peak at 23.2 min) and dehydrotomatine (peak at 22.4 min). (D) Quantification of α -tomatine and dehydrotomatine contents using LC-MS. Error bars indicate standard error ($n=3$).

yield tomatidine. Similar result was obtained in case of co-incubation of dehydrotomatidine with *SIS5 α R1* and *Sl3 β HSD1* (Figure 5B), and this result was consistent with our previous work (Lee et al. 2019). Taken together, either *SIS5 α R2* or *SIS5 α R1*, in combination with *Sl3 β HSD1* and cofactors, could reconstitute the conversion of dehydrotomatidine to tomatidine in an in vitro assay.

CRISPR/Cas9-mediated genome editing of *SIS5 α R1* or *SIS5 α R2* in tomato hairy roots

To verify the contribution of *SIS5 α R1* and *SIS5 α R2* in α -tomatine biosynthesis in vivo, *SIS5 α R1* and *SIS5 α R2* were each independently disrupted using CRISPR/Cas9-

mediated genome editing in tomato hairy roots. In this study, we used a CRISPR/Cas9 binary vector designated as pMgP237-2A-GFP that permits expression of multiplex gRNAs by a pre-tRNA processing mechanism (Hashimoto et al. 2018; Nakayasu et al. 2018). To design the gRNAs specific to *SIS5 α R1* and to *SIS5 α R2*, we conducted in silico analysis using Design sgRNAs for CRISPRko and Cas-OT software (Xiao et al. 2014). Then, the selected target sequences were inserted into a pMgP237-2A-GFP backbone vector, and we constructed a targeting vector specific to *SIS5 α R1* and one specific to *SIS5 α R2*, designated as pMgP237-*SIS5 α R1*ko and pMgP237-*SIS5 α R2*ko, respectively (Supplementary Figure S1). The hypocotyls of tomato cv. Micro-Tom

were infected with *A. rhizogenes* harboring each of the two constructed vectors. Several lines of transgenic hairy roots were obtained, and the mutations in the region surrounding the target sites of gRNAs were analyzed.

Among the pMgP237-SIS5 α R1ko transgenic hairy roots, a formation of a heteroduplex, which indicates the mutation at the target site, was detected as shifted peaks in #1 and #6 (Supplementary Figure S2). Among the pMgP237-SIS5 α R2ko transgenic hairy roots, deletion of a large segment or a formation of a heteroduplex was detected in #5, #7, #8, #10, #11, and #12 (Supplementary Figure S2). To confirm the mutations at the target sites, four lines for each of pMgP237-SIS5 α R1ko (lines #1 and #6) and pMgP237-SIS5 α R2ko (line #7 and #12) were selected, and sequencing analyses were conducted. The lines #1 and #6 for pMgP237-SIS5 α R1ko had no intact *SIS5 α R1* sequences, and all of the sequences showed the translational frameshift mutations (Figure 6A). In the lines #7 and #12 for pMgP237-SIS5 α R2ko, all the detected sequences showed some deletions in the *SIS5 α R2* gene (Figure 6A).

Endogenous SGAs of these selected lines were extracted and analyzed by LC-MS. The level of α -tomatine in the pMgP237-SIS5 α R1ko_#1 and #6 was similar to that in the vector control, and the amounts of dehydrotomatine were not affected (Figure 6B–D). On the other hand, the α -tomatine level was significantly decreased in the pMgP237-SIS5 α R2ko_#7 and #12, and a corresponding increase in dehydrotomatine level was detected as compared with that in the vector control (Figure 6B–D). These results indicate that *SIS5 α R2* is involved in the conversion of dehydrotomatidine to tomatidine in tomato, while *SIS5 α R1* is not responsible for biosynthesis of SGAs.

Discussion

In the present study, we found that *SIS5 α R1* and *SIS5 α R2* catalyze the conversion of tomatid-4-en-3-one to tomatid-3-one in vitro (Figure 5A). In the *SIS5 α R2*-knockout tomato hairy roots, α -tomatine was dramatically decreased and corresponding amounts of dehydrotomatine were accumulated, while the disruption of *SIS5 α R1* did not affect the α -tomatine and dehydrotomatine levels (Figure 6). These results clearly demonstrate that *SIS5 α R2*, but not *SIS5 α R1*, is involved in the C5 α reduction step of the metabolic conversion of dehydrotomatidine to tomatidine in α -tomatine biosynthesis.

Arabidopsis DET2 (*AtDET2*) is the first gene isolated in plants coding functional steroid 5 α -reductase, and the *Arabidopsis det2-1* mutant shows a dwarf and de-etiolated phenotype because of a deficiency of bioactive brassinosteroids (Noguchi et al. 1999). In plants, the reduction of the steroid $\Delta^{4,5}$ double bond is

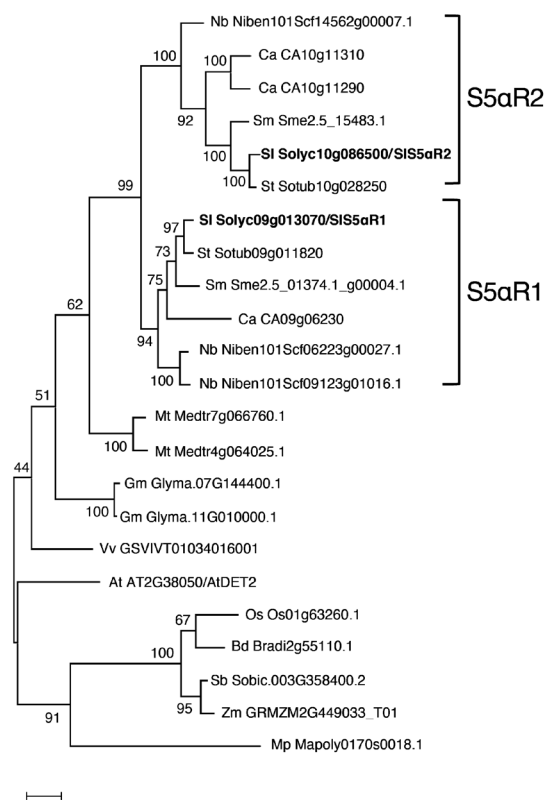


Figure 7. Phylogenetic analysis of plant *AtDET2* homologs. Species were represented tomato (Sl), potato (St), eggplant (Sm), *Capsicum annuum* (Ca), *Nicotiana benthamiana* (Nb), *Brachypodium distachyon* (Bd), soybean (Gm), *Medicago truncatula* (Mt), rice (Os), *Sorghum bicolor* (Sb), *Vitis vinifera* (Vv), and maize (Zm). A phylogenetic tree was generated using the maximum likelihood method in MEGA7. Bootstrap values based on 1,000 replicates are shown at the branching points.

an important reaction in the biosynthesis of the plant hormone BRs since all known bioactive BRs lack $\Delta^{4,5}$ double bond (Rosati et al. 2005). Steroid 5 α -reductase is likely to be conserved in plant kingdom, and we found that some plant species have multiple *S5 α R* genes in their genome. We found that all Solanaceae species analyzed have two or more *S5 α R* genes in the genome, and phylogenetic analysis demonstrated that the *S5 α R* proteins of solanaceous plants are clearly divided into two distinguishable clades (Figure 7), suggesting that duplication of the ancestral *S5 α R* gene has occurred in solanaceous plants. *SIS5 α R1* shows higher homology to *AtDET2*, suggesting that *SIS5 α R1* would keep to function only in the biosynthesis of phytosterols and brassinosteroids. Then, the duplication gives rise to *SIS5 α R2*, which is specialized in α -tomatine biosynthesis. The duplication and neofunctionalization have been reported for several enzymes involved in phytosterols, BRs and cholesterol biosynthesis (Christ et al. 2019; Sawai et al. 2014; Sonawane et al. 2017). For instance, potato and tomato have two DWF1 homologs named SSR1 and SSR2 (Sawai et al. 2014). SSR1 corresponds

to DWF1 and has $\Delta^{24(28)}$ reduction activity required for phytosterol biosynthesis, while SSR2 catalyzes $\Delta^{24(25)}$ reduction of cycloartenol and desmosterol for cholesterol biosynthesis. Our in vitro assay revealed that both SIS5 α R1 and SIS5 α R2 encode the functional steroid 5 α -reductase and catalyze the conversion of 5 α -reduction of tomatid-4-en-3-one to yield tomatid-3-one (Figure 5A). Furthermore, in combination with Sl3 β HSD1, each of SIS5 α R1 and SIS5 α R2 could complete the four reaction steps converting dehydrotomatidine to tomatidine in the in vitro assays (Figure 5B). These observations suggest that the different physiological roles for SIS5 α R1 and SIS5 α R2 reflect factors other than neofunctionalization of catalytic activity.

Recently, Thagun et al. (2016) reported that *Jasmonate-Responsive ERF 4 (JRF4)* comprehensively regulates α -tomatine biosynthetic genes, including those involved in the upstream mevalonate pathway, in tomatoes. Quantitative RT-PCR analysis in various tissues of tomatoes showed different expression patterns between SIS5 α R1 and SIS5 α R2, and the expression pattern of SIS5 α R2 is in good agreement with those of known SGA biosynthetic genes. Therefore, our results indicate that acquisition of the transcriptional regulation, which cooperates with other SGA biosynthetic genes, contributes to functional specialization of SIS5 α R2 for SGA biosynthesis. However, our quantitative RT-PCR analyses and RNA-seq data of the Tomato Functional Genomics Database (<http://ted.bti.cornell.edu/>) demonstrated that SIS5 α R1 is also expressed in certain amounts in SGA containing tissues, suggesting that there are mechanisms, in addition to transcriptional control, to explain the functional differentiation of SIS5 α R1 and SIS5 α R2 in vivo. For example, post-translational regulation such as distinct intracellular localization and/or protein–protein interaction in the metabolon can be one of the mechanisms, as discussed below.

SIS5 α R2-knockout tomato hairy roots contained very little α -tomatine, and the amount of dehydrotomatine increased to a level comparable to the level of α -tomatine accumulated in control tomato hairy roots (Figure 6). Dehydrotomatine is biosynthesized via glycosylation from dehydrotomatidine, which is not a direct substrate of SIS5 α R2. Dehydrotomatidine is metabolized via oxidation and isomerization by Sl3 β HSD1 to yield tomatid-4-en-3-one, which is a direct substrate of SIS5 α R2 (Figure 1). Considering that isomerase reaction of mammalian 3 β HSD has been reported to be irreversible (Thomas et al. 2005), high accumulation of dehydrotomatine suggests that Sl3 β HSD1 does not function well in the SIS5 α R2-knockout tomato hairy roots. This may be the reason why dehydrotomatine but not tomatid-4-en-3-one accumulated in the SIS5 α R2-knockout tomato hairy roots. It has been reported that protein–protein interaction that allows the formation

of protein complexes of sequential metabolic enzymes, termed metabolon, enhances direct channeling of substrates between the biosynthetic enzymes, providing increased control over metabolic pathway fluxes (Obata 2019). So far, to the best of our knowledge, there has been no report of metabolon formation in SGA biosynthesis, but the physical protein–protein interaction of SIS5 α R2 with Sl3 β HSD1, or other SGA biosynthetic enzymes, may influence the functional differentiation of SIS5 α R1 and SIS5 α R2. In order to address this hypothesis, we need to examine in detail the subcellular localization of SIS5 α R2 and other SGA biosynthetic enzymes, as well as protein–protein interactions among SGA biosynthetic enzymes.

In conclusion, we showed here that SIS5 α R2 is a key enzyme in the production of tomatidine from dehydrotomatidine in tomatoes, thereby contributing to structural diversity of SGAs in the Solanaceae family. SGA is well known as a toxic and antinutritional substance in a Solanaceae crop, but SGAs are considered to play protective roles against plant pathogens and predators for the plants. Through this study, it may be possible to clarify the significance of solanaceous plants, producing SGA with various structures.

Acknowledgements

We thank Mina Yamamoto for technical assistance.

This study was supported by the Grant-in-Aid for Japan Society for the Promotion of Science (JSPS) Fellows.

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