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

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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, C5α reduction, and C3 reduction. Our previous studies (Lee et al. 2019) revealed that Sl3αHSD is involved in the three reactions except for C5α reduction, and in the present study, we aimed to elucidate the gene responsible for the C5α reduction step in the conversion of dehydrotomatidine to tomatidine. We characterized the two genes, SlS5αR1 and SlS5αR2, which show high homology with SGA biosynthetic genes, while SlS5αR1 is ubiquitously expressed, suggesting the involvement of SlS5αR2 in SGA biosynthesis. Biochemical analysis of the recombinant proteins revealed that both of SlS5αR1 and SlS5αR2 catalyze α-reduction of β-estradiol 3,17-dione (β-estradiol 5α-reductase of Arabidopsis thaliana). The expression pattern of SlS5αR2 is similar to those of SGA biosynthetic genes, while SlS5αR1 is ubiquitously expressed, suggesting the involvement of SlS5αR2 in SGA biosynthesis. Biochemical analysis of the recombinant proteins revealed that both of SlS5αR1 and SlS5αR2 catalyze the reduction of tomatid-4-en-3-one at C5α to yield tomatid-3-one. Then, SlS5αR1- or SlS5αR2-knockout hairy roots were constructed using CRISPR/Cas9 mediated genome editing. In the SlS5αR2-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 SlS5αR1-knockout hairy roots. These results indicate that SlS5αR2 is responsible for the C5α reduction in α-tomatine biosynthesis and that SlS5αR1 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...
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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 monoxygenases (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 dehydrotomatine as major SGAs (Friedman 2002). α-Tomatine and dehydrotomatine are biosynthesized via four steps of glycosylations from their respective aglycones, tomatidine and dehydrotomatidine. Tomatidine possesses a single bond between C5 and C6 (dihydro type), while dehydrotomatidine has a Δ5,6 double bond (dehydro type), and is biosynthesized from dehydrotomatidine by the four-step reaction process shown in Figure 1. We recently identified that the gene designated as Sl3βHSD1 is involved in the conversion of dehydrotomatidine to tomatidine (Lee et al. 2019). Sl3βHSD1 is a multifunctional enzyme that possesses the activities of 3β-hydroxysteroid dehydrogenase (3βHSD) and 3-ketosteroid isomerase (3KSI) when acting on dehydrotomatidine 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, Sl3βHSD1 is involved in the three reaction steps among the four reactions from dehydrotomatidine 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 (S5αR), which is involved in the NADPH-dependent reduction of the Δ5,6 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 S5αR protein that shares significant sequence identity with mammalian S5αRs (Noguchi et al. 1999). AtDET2 catalyzes the NADPH-dependent reduction of the A4 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, αSlS5αR1, which is an ortholog of mammalian S5αR1 and αSlS5αR2 using progesterone as a substrate has remained unknown.

In the present study, we isolated a second DET2 homolog from tomato, designated as αSlS5αR2. The expression pattern of αSlS5αR1 and αSlS5αR2 using high-performance liquid chromatography (HPLC). α-Tomatine was purchased from Sigma-Aldrich.

**Materials and methods**

**Plant materials**

The tomato plant used in this study was S. lycopersicum cv Micro-Tom (TOMJPF00001), obtained from the NBRP (MEXT, Co., Ltd. (Tokyo, Japan), and 5α-pregnane-3,20-dione was purchased from Santa Cruz Biotechnology (Santha Cruz, CA, USA). Dehydrotomatidine was isolated in our lab from tomatidine purchased from Chromadex (Irvine, CA, USA) using high-performance liquid chromatography (HPLC).

**RNA extraction and reverse transcription**

Total RNAs were extracted using the RNaseasy 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 GeneAce SYBR® qPCR Mix α (Nippon Gene), with the following primers 1 and 2 for αSlS5αR1, 3 and 4 for αSlS5αR2, 5 and 6 for ubiquitin (Supplementary Table S2). 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 αSlS5αR1, αSlS5αR1 and αSl3βHSD1**

The DNA fragments containing the ORF of Solyc09g013070 (αSlS5αR1) and Solyc10g086500 (αSlS5αR2) were isolated using the cDNA template from the tomato leaves with primer sets 7 and 8 for αSlS5αR1, 9 and 10 for αSlS5αR2 (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).

**Heterologous expression of the recombinant αSlS5αR1, αSlS5αR1 and αSl3βHSD1 proteins**

The DNA fragments containing the ORF of αSlS5αR1 and αSlS5αR2 and αSl3βHSD1 proteins were amplified using PCR with the clone as a template and primer sets 11 and 12 (Supplementary Table S2).
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SLSSαR2 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 slssαR1 and slssαR2 ORFs and transfection of Spodoptera frugiperda 9 insect cells were carried out according to the manufacturer's instructions (Life Technologies). Heterologous expression of slssαR1 and slssαR2 proteins in insect cells was conducted as described by Ohnishi et al. (2006). Similarly, the DNA fragments containing the sl3βhSD1 gene ORF were ligated into pCold ProS2 vector (TaKaRa), and recombinant sl3βHSD1 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 activity assay of recombinant sl3βHSD1 protein, and the assay was performed using 100µl of reaction mixture comprising 100mM bis-tris-HCl (pH 7.2), 2.5mM NAD⁺ as a coenzyme, 50µM dehydrotomatidine as a substrate, and the purified sl3βHSD1. The enzymatically synthesized tomatid-4-en-3-one was further metabolized by recombinant sl5sr1 to produce tomatid-3-one, and the reaction was carried out as described below.

Microsomal fractions of the insect cells expressing sl5sr1 or sl5sr2 were obtained from 100ml of cultured cell suspension. Infected cells were washed with phosphate-buffered saline and suspended in buffer A, consisting of 20mM potassium phosphate (pH 7.5), 20% (v/v) glycerol, 1mM ethylenediaminetetraacetic acid, and 1mM 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 sl5sr1 and sl5sr2 for steroid C5 reduction were performed using 100µl reaction mixture comprising 100mM potassium phosphate (pH 7.2), 1mM NADPH or NADH as a coenzyme, the enzymatically synthesized tomatid-4-en-3-one, and the microsomal fraction containing sl5sr1 or sl5sr2. The conversion of dehydrotomatidine to tomatidine was reconstituted in an in vitro assay by mixing the purified sl3βHSD1 protein with sl5sr1 or sl5sr2-containing microsomes. The reaction mixture consisted of 100µM bis-tris-HCl buffer (pH 7.2), purified sl3βHSD1 protein, microsomes containing sl5sr1 or sl5sr2, 2.5mM NAD⁺, 1mM NADPH, and 25µM dehydrotomatidine. All the reactions were performed at 30°C for 2h and were terminated by the addition of 100µl 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 (30m×0.25mm, 0.25µm 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 5α-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 200µl 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 (1.8µm, 2.1×100mm²; 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 sl5sr1 and sl5sr2 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 sl5sr1 and sl5sr2, we conducted in silico analyses using the Web tool Design sgRNAs for CRISPRko (https://portals.broadinstitute.org/ppp/public/analysis-tools/sgrna-design) and Cas-OT software (Xiao et al. 2014). Then we selected target sequences named sl5sr1_492-511 and sl5sr1_552-571 in the coding region of sl5sr1 and sl5sr2_449-468 and sl5sr2_511-530 in the coding region of sl5sr2, respectively. To enhance the efficiency of gRNA transcription from U6 promoter, one G was added to the 5’-end of sl5sr1_492-511, sl5sr1_552-571 and sl5sr2_511-530. Two DNA fragments composed of the gRNA scaffold and tRNA scaffold between two target sequences, sl5sr1_492-511/slr5sr1_552-571 and sl5sr2_449-468/sl5sr2_511-530, were generated by PCR using pMD-gtRNA containing gRNA and tRNA scaffolds as a template and primer sets containing restriction enzyme Bsal sites (13 and 14 for sl5sr1_492-511/ sl5sr1_552-571, 15 and 16 for sl5sr2_449-468/sl5sr2_511-530) (Supplementary Table S2). The units containing two gRNAs–tRNAs were then independently inserted into the Bsal site of pMgP237-2A-GFP using Golden Gate Cloning methods to construct the CRIPR/Cas9 vectors, labeled pMgP237-sl5sr1ko and pMgP237-sl5sr2ko, respectively.

Generation of sl5sr1- and sl5sr2-disrupted transgenic tomato hairy roots
The vectors pMgP237-sl5sr1ko and pMgP237-sl5sr2ko 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.
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-SlS5αR1ko transformants and primers 21 and 22 for pMgP237-SlS5αR2ko 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-SlS5αR1ko_#1 and #6, pMgP237-SlS5αR2ko_#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 SlS5αR1, which has been already characterized to encode a functional steroid 5α-reductase (Lee et al. 2019), and

Figure 2. Multiple sequence alignment of SlS5αR1 and SlS5αR2 with AtDET2 from *Arabidopsis thaliana* and human hSSαR1. 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.
Soly10g086500 was identical to LcDET2, which was previously characterized to encode a functional steroid 5α-reductase (Rosati et al. 2005), and here we designated it as SIS5αR2. SIS5αR1 and SIS5αR2 show 63% and 57% amino acid identity to AtDET2, respectively, and share 67% amino acid identity to each other. Both SIS5αR1 and SIS5αR2 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 5α-reductase (Figure 2; Russell and Wilson 1994). Both SIS5αR1 and SIS5αR2 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 SIS5αR1 and SIS5αR2 in various tissues of tomato**

The expression of SIS5αR1 and SIS5αR2 was analyzed in the RNA-seq data of the Tomato Functional Genomics Database (http://ted.bti.cornell.edu/). SIS5αR2 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 SIS5αR1 remained high even in red ripe fruits where the expression of SGA genes was low (Supplementary Table S1). To confirm the expression of SIS5αR1 and SIS5αR2, real-time PCR was performed using the reverse-transcription products of total RNAs extracted from various tissues of *S. lycopersicum* cv. Micro-Tom. SIS5αR2 was highly expressed in flowers, where a large amount of α-tomatine is accumulated (Roddick 1974), while the expression level of SIS5αR2 was low in red mature fruits (Figure 3), in which α-tomatine is further metabolized to esculeotide A and the α-tomatine expression level is very low (Iijima et al. 2009). This expression pattern of SIS5αR2 is similar to those of the SGA biosynthetic genes Sl16DOX, GAME1, and Sl3βHSD1 (Lee et al. 2019; Nakayasu et al. 2017). On the other hand, SIS5αR1 was abundantly expressed in red mature fruits, and the expression pattern of SIS5αR1 is in contrast to that of the known SGA biosynthetic genes (Figure 3).

**In vitro functional analysis of the C5α-reduction activity of SIS5αR2 on tomatid-4-en-3-one to tomatid-3-one**

In our recent studies, the recombinant SIS5αR1 protein was expressed with a baculovirus-mediated expression system in *Spodoptera frugiperda* 9 insect cells and confirmed to catalyze C5α-reduction of tomatid-4-en-3-one to yield tomatid-3-one in vitro (Lee et al. 2019). Previously, it was reported that recombinant SIS5αR2 expressed in mammalian cells (COS-7) was active on typical substrates of mammalian 5αRs (progesterone, testosterone and androstenedione) (Rosati et al. 2005). In the present study, to compare the catalytic activities of SIS5αR1 and SIS5αR2, the recombinant SIS5αR2 was prepared with a baculovirus-mediated expression system and C5-reduction activities were analyzed. First, the functional expression of SIS5α2 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 5α-pregnane-3,20-dione in terms of both the retention time and the mass spectrum (Figure 4). Although SIS5αR2 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 SIS5αR2 in the baculovirus-insect cell system was confirmed.

We evaluated the C5α-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 Sl3βHSD1 and SIS5αR1 (Lee et al. 2019). Tomatid-4-en-3-one was synthesized from dehydrotomatidine by enzymatic conversion with the recombinant Sl3βHSD1 using NAD⁺ as a coenzyme, and tomatid-3-one was synthesized from tomatid-4-en-3-one by enzymatic conversion with SIS5αR1 using NADPH as a coenzyme.
Then, the recombinant SlS5\textalpha\textalphaR2 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 SlS5\textalpha\textalphaR2 was identical to tomatid-3-one, obtained by incubating tomatid-4-en-3-one with SlS5\textalpha\textalphaR1 (Figure 5A). Thus, SlS5\textalpha\textalphaR2 could catalyze the reduction of tomatid-4-en-3-one at C5\textalpha to yield tomatid-3-one.

Next, we conducted a co-incubation assay of SlS5\textalpha\textalphaR2 with Sl3\textbetaHSD1 using dehydrotomatidine as a substrate, and the reaction products were analyzed by LC-MS (Figure 5B). When incubating dehydrotomatidine with Sl3\textbetaHSD1, 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 SlS5\textalpha\textalphaR2 resulted in the detection of two major products with the retention times of 20.4 and 22.4 min, respectively (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 SlS5\textalpha\textalphaR2 and Sl3\textbetaHSD1, dehydrotomatidine was first converted to tomatid-4-en-3-one by Sl3\textbetaHSD1, and SlS5\textalpha\textalphaR2 subsequently catalyzed the reduction of tomatid-4-en-3-one at C5\textalpha to form tomatid-3-one, and Sl3\textbetaHSD1 finally catalyzed the reduction of tomatid-3-one at C3 to

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Figure 4. In vitro assay of the C5 reduction activity of SlS5\textalphaR1 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 5a-pregnan-3,20-dione was monitored at m/z 231 and is shown in gray. (B) Mass spectrum of the (a) authentic 5a-pregnan-3,20-dione at a retention time of 9.2 min and (b) product peak of progesterone incubated with SlS5\textalphaR2 and NADPH.

Figure 5. In vitro assays of SlS5\textalphaR1 and SlS5\textalphaR2 with tomatid-4-en-3-one. (A) GC-MS analysis of the in vitro assays of SlS5\textalphaR1/2 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 SlS5\textalphaR1/2 and Sl3\textbetaHSD1 incubated with dehydrotomatidine as a substrate. Substrate and products were monitored by SIM mode at m/z 412, 414, and 416.
yield tomatidine. Similar result was obtained in case of co-incubation of dehydrotomatidine with SlS5αR1 and Sl3βHSD1 (Figure 5B), and this result was consistent with our previous work (Lee et al. 2019). Taken together, either SlS5αR1 or SlS5αR2, in combination with Sl3βHSD1 and cofactors, could reconstitute the conversion of dehydrotomatidine to tomatidine in an in vitro assay.

**CRISPR/Cas9-mediated genome editing of SlS5αR1 or SlS5αR2 in tomato hairy roots**

To verify the contribution of SlS5αR1 and SlS5αR2 in α-tomatine biosynthesis in vivo, SlS5αR1 and SlS5αR2 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 SlS5αR1 and to SlS5αR2, 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 SlS5αR1 and one specific to SlS5αR2, designated as pMgP237-SlS5αR1ko and pMgP237-SlS5αR2ko, 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 CRNAs were analyzed. Among the pMgP237-SSL5αR1ko 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-SSL5αR2ko 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-SSL5αR1ko (lines #1 and #6) and pMgP237-SSL5αR2ko (line #7 and #12) were selected, and sequencing analyses were conducted. The lines #1 and #6 for pMgP237-SSL5αR1ko had no intact SSL5αR1 sequences, and all of the sequences showed the translational frameshift mutations (Figure 6A). In the lines #7 and #12 for pMgP237-SSL5αR2ko, all the detected sequences showed some deletions in the SSL5αR2 gene (Figure 6A).

Endogenous SGAs of these selected lines were extracted and analyzed by LC-MS. The level of α-tomatine in the pMgP237-SSL5αR1ko_#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-SSL5αR2ko_#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 SSL5αR2 is involved in the conversion of dehydrotomatidine to tomatidine in tomato, while SSL5αR1 is not responsible for biosynthesis of SGAs.

**Discussion**

In the present study, we found that SSL5αR1 and SSL5αR2 catalyze the conversion of tomatid-4-en-3-one to tomatid-3-one in vitro (Figure 5A). In the SSL5αR2-knockout tomato hairy roots, α-tomatine was dramatically decreased and corresponding amounts of dehydrotomatine were accumulated, while the disruption of SSL5αR1 did not affect the α-tomatine and dehydrotomatine levels (Figure 6). These results clearly demonstrate that SSL5αR2, but not SSL5αR1, is involved in the C5α 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 5α-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 Δ^{4,5} double bond is an important reaction in the biosynthesis of the plant hormone BRs since all known bioactive BRs lack Δ^{4,5} double bond (Rosati et al. 2005). Steroid 5α-reductase is likely to be conserved in plant kingdom, and we found that some plant species have multiple S5αR genes in their genome. We found that all Solanaceae species analyzed have two or more S5αR genes in the genome, and phylogenetic analysis demonstrated that the S5αR proteins of solanaceous plants are clearly divided into two distinguishable clades (Figure 7), suggesting that duplication of the ancestral S5αR gene has occurred in solanaceous plants. SSL5αR1 shows higher homology to *AtDET2*, suggesting that SSL5αR1 would keep to function only in the biosynthesis of phytosterols and brassinosteroids. Then, the duplication gives rise to SSL5αR2, 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 Δ24(28) reduction activity required for phytosterol biosynthesis, while SSR2 catalyzes Δ24(25) reduction of cycloartenol and desmosterol for cholesterol biosynthesis. Our in vitro assay revealed that both SlS5αR1 and SlS5αR2 encode the functional steroid 5α-reductase and catalyze the conversion of 5α-reduction of tomatid-4-en-3-one to yield tomatid-3-one (Figure 5A). Furthermore, in combination with Sl3βHSD1, each of SlS5αR1 and SlS5αR2 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 SlS5αR1 and SlS5αR2 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 SlS5αR1 and SlS5αR2, and the expression pattern of SlS5αR2 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 SlS5αR2 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 SlS5αR1 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 SlS5αR1 and SlS5αR2 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.

SlS5αR2-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 SlS5αR2. Dehydrotomatidine is metabolized via oxidation and isomerization by Sl3βHSD1 to yield tomatid-4-en-3-one, which is a direct substrate of SlS5R2 (Figure 1). Considering that isomerase reaction of mammalian 3βHSD has been reported to be irreversible (Thomas et al. 2005), high accumulation of dehydrotomatine suggests that Sl3βHSD1 does not function well in the SlS5αR2-knockout tomato hairy roots. This may be the reason why dehydrotomatine but not tomatid-4-en-3-one accumulated in the SlS5αR2-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 SlS5αR2 with Sl3βHSD1, or other SGA biosynthetic enzymes, may influence the functional differentiation of SlS5αR1 and SlS5αR2. In order to address this hypothesis, we need to examine in detail the subcellular localization of SlS5αR2 and other SGA biosynthetic enzymes, as well as protein–protein interactions among SGA biosynthetic enzymes.

In conclusion, we showed here that SlS5αR2 is a key enzyme in the production of tomatidine from dehydrotomatidine in tomatines, thereby contributing to structural diversity of SGA 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.

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