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Identification of α-Tomatine 23-Hydroxylase Involved in the Detoxification of a Bitter Glycoalkaloid

Masaru Nakayasu[1,7], Ryota Akiyama[1], Midori Kobayashi[1], Hyoung Jae Lee[1], Takashi Kawasaki[1,7], Bunta Watanabe[2], Shingo Urakawa[1], Junpei Kato[1], Yukihiro Sugimoto [1], Yoko Iijima[3], Kazuki Saito[4,5], Toshiya Muranaka [6], Naoyuki Umemoto[5] and Masaharu Mizutani[1,*]

[1]Graduate School of Agricultural Science, Kobe University, Rokkoudai 1-1, Nada-ku, Kobe, Hyogo, 657-8501 Japan
[2]Institute for Chemical Research, Kyoto University, Uji, Kyoto, 611-0011 Japan
[3]Department of Nutrition and Life Science, Kanagawa Institute of Technology, 1030 Shimo-ogino, Atsugi, Kanagawa, 243-0292 Japan
[4]Graduate School of Pharmaceutical Sciences, Chiba University, Inohana 1-8-1, Chuo-ku, Chiba, 260-8675 Japan
[5]RIKEN Center for Sustainable Resource Science, Suehiro-cho 1-7-22, Tsurumi-ku, Yokohama, Kanagawa, 230-0045 Japan
[6]Department of Biotechnology, Graduate School of Engineering, Osaka University, Yamadaoka 2-1, Suita, Osaka, 565-0871 Japan
[7]Present address: Laboratory of Plant Gene Expression, Research Institute for Sustainable Humanosphere, Kyoto University, Gokasho, Uji, Kyoto, 611-0011 Japan.
*Corresponding author: E-mail, mizutani@gold.kobe-u.ac.jp; Fax, +81-78-803-5884.
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Tomato plants (*Solanum lycopersicum*) contain steroidal glycoalkaloid α-tomatine, which functions as a chemical barrier to pathogens and predators. α-Tomatine accumulates in all tissues and at particularly high levels in leaves and immature green fruits. The compound is toxic and causes a bitter taste, but its presence decreases through metabolic conversion to nontoxic esculeoside A during fruit ripening. This study identifies the gene encoding a 23-hydroxylase of α-tomatine, which is a key to this process. Some 2-oxoglutarate-dependent dioxygenases were selected as candidates for the metabolic enzyme, and *Solyc02g062460*, designated *Sl23DOX*, was found to encode α-tomatine 23-hydroxylase. Biochemical analysis of the recombinant Sl23DOX protein demonstrated that it catalyzes the 23-hydroxylation of α-tomatine and the product spontaneously isomerizes to neorickiioside B, which is an intermediate in α-tomatine metabolism that appears during ripening. Leaves of transgenic tomato plants overexpressing *Sl23DOX* accumulated not only neorickiioside B but also another intermediate, lycoperoside C (23-*O*-acetylated neorickiioside B). Furthermore, the ripe fruits of *Sl23DOX*-silenced transgenic tomato plants contained lower levels of esculeoside A but substantially accumulated α-tomatine. Thus, Sl23DOX functions as α-tomatine 23-hydroxylase during the metabolic processing of toxic α-tomatine in tomato fruit ripening and is a key enzyme in the domestication of cultivated tomatoes.

Keywords: 2-Oxoglutarate-dependent dioxygenase • α-Tomatine • Fruit ripening • Sl23DOX • Steroidal glycoalkaloid • Tomato (*Solanum lycopersicum*).

Accession numbers: The nucleotide sequence of *Sl23DOX* reported in this article has been submitted to DDBJ under accession number LC508578.

Introduction

Steroidal glycoalkaloids (SGAs) are widely found in the genus *Solanum*, which includes several important crops, such as cultivated tomato (*Solanum lycopersicum*), potato (*Solanum tuberosum*) and eggplant (*Solanum melongena*) (Friedman 2002, Friedman 2006, Mennella et al. 2010). SGAs are toxic to various organisms, such as bacteria, fungi, viruses, insects, animals and humans (Friedman 2002, Friedman 2006, Milner et al. 2011). In tomato plants, α-tomatine, which is a well-known tomato SGA, is contained in all tissues and at particularly high levels in leaves and immature fruits (Friedman 2002, Kozukue et al. 2004, Cataldi et al. 2005). In contrast, another SGA, esculeoside A, accumulates in the ripe fruits (Fujiwara et al. 2004, Nohara et al. 2010). The α-tomatine contents decrease, whereas the esculeoside A contents increase during fruit ripening (Iijima et al. 2008). In addition, Iijima et al. (2009) reported that the production and perception of ethylene are correlated with the change in levels of α-tomatine and esculeoside A in fruits, suggesting that α-tomatine is metabolized to esculeoside A during ripening.

The SGA content profiles in the fruits of several tomato species show a variety of patterns. For example, the bitter-flavored accession LA2213 of *S. lycopersicum* var. *cerasiforme* possesses α-tomatine at high levels even in ripe fruits, whereas α-tomatine is not detected in the sweet-flavored accession LA2295 (Rick et al. 1994). In addition, a 90% association between high α-tomatine content and bitter flavor was found among 88 accessions of var. *cerasiforme*, indicating that α-tomatine is the source of bitter flavor. Iijima et al. (2013) demonstrated that the ripe fruits of wild tomato accessions LA0716 (*Solanum pennellii*), LA1414 (*Solanum cheesmaniae*) and LA1777 (*Solanum habrochaites*), in addition to LA2213 (var. *cerasiforme*), contain a high ratio of α-tomatine to total SGA, although those of LA1090 (*S. lycopersicum* cv. Rutgers),

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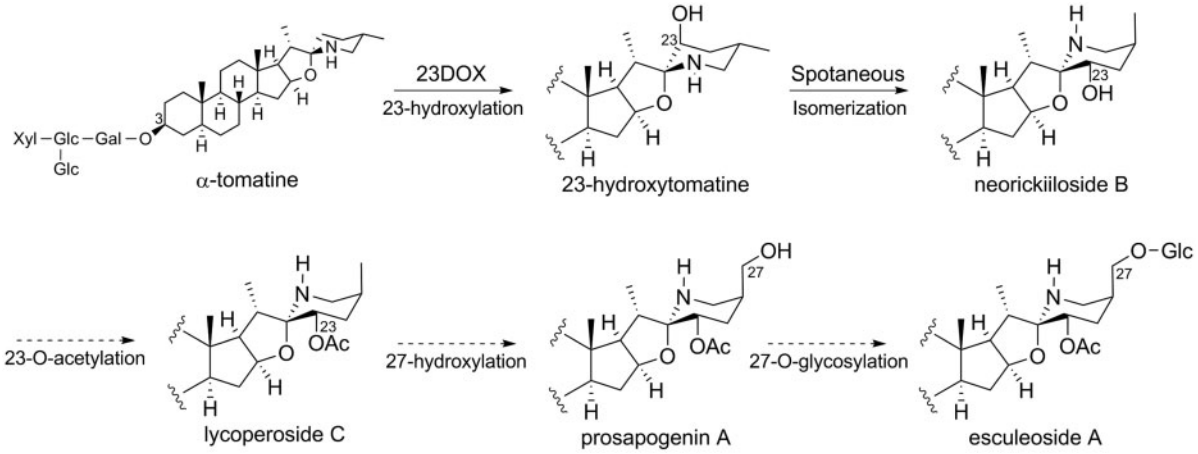

Fig. 1 The putative pathway for the metabolism of α-tomatine to esculeoside A during tomato fruit ripening. The solid arrows indicate the reaction step suggested in this study. Dashed arrows represent reaction stages that remain unclear.

LA3911 (*S. lycopersicum* cv. Micro-Tom) and LA1589 (*Solanum pimpinellifolium*) abundantly accumulate esculeoside A.

SGAs are composed of a C_{27} steroid containing a nitrogen atom with an oligosaccharide on the hydroxy group at the C3 position. In α-tomatine, lycotetraose is linked to the C3 hydroxy group of the spirosolane-type aglycone tomatidine (**Fig. 1**). SGAs are biosynthesized from cholesterol (Sawai et al. 2014), which is subsequently modified through multiple reaction steps including hydroxylation, transamination, E- and F-ring closure and transglycosylation (Petersen et al. 1993, Friedman 2006, Ginzberg et al. 2009). Previously, three cytochrome P450 monooxygenases were reported to be responsible for SGA biosynthesis in potato and tomato (Itkin et al. 2013, Umemoto and Sasaki 2013, Umemoto et al. 2016). In addition, several UDP-glycosyltransferases (UGTs) involved in the glycosylation steps of SGA biosynthesis were identified in potato and tomato (Moehs et al. 1997, McCue et al. 2005, McCue et al. 2006, McCue et al. 2007, Itkin et al. 2011, Itkin et al. 2013). Recently, we identified and characterized a 2-oxoglutarate-dependent dioxygenase (DOX) named 16DOX, which catalyzes 16α-hydroxylation of (22*S*)-22,26-dihydroxycholesterol during the early stages of SGA biosynthesis in potato and tomato (Nakayasu et al. 2017). In tomato fruits, α-tomatine is thought to be modified further by hydroxylations at C-23 and C-27, acetylation at the C-23 hydroxy group and glucosylation at the C-27 hydroxy group, resulting in conversion to esculeoside A during fruit ripening (Iijima et al. 2008). Iijima et al. (2013) identified three candidate intermediates, neorickiioside B, lycoperoside C and prosapogenin A, in the metabolic pathway (**Fig. 1**). Gene silencing of *Solyc06g062290* and *Solyc10g085230*, which encode UGTs, causes an increase in α-tomatine levels and a change in the contents of other SGAs, such as esculeoside A (Alseekh et al. 2015). However, the genes that are involved in the metabolic conversion of α-tomatine to esculeoside A remain to be elucidated.

In this study, we explored the gene involved in the first step of α-tomatine catabolism, namely a 23-hydroxylase of α-tomatine. Since we previously characterized 16DOX responsible for SGA biosynthesis, we selected three DOX candidates responsible for the hydroxylation of α-tomatine from the tomato public databases. One of the candidates, *Solyc02g062460* named *Sl23DOX*, is identified to encode α-tomatine 23-hydroxylase involved in the first step in the metabolic conversion of α-tomatine to esculeoside A during tomato fruit ripening.

Results

Selection of candidate genes for a C23-hydroxylase of α-tomatine

The content of α-tomatine is high in immature green fruits but decreases dramatically during fruit ripening, whereas the content of esculeoside A increases in inverse proportion to the decrease in α-tomatine (Iijima et al. 2008). The first and key enzyme in this metabolic conversion from α-tomatine to esculeoside A is thought to be an α-tomatine 23-hydroxylase. In our previous work, we characterized 16DOX, a member of the DOX superfamily, that catalyzes 16α-hydroxylation of steroid involved in SGA biosynthesis in potato and tomato (Nakayasu et al. 2017). Therefore, we focused on the genes encoding the DOX superfamily to find candidates for α-tomatine 23-hydroxylase. A total of 239 DOX transcripts were extracted using the BLASTP search of peptide sequences encoded by tomato genes available from the genome databases of *Solanum* species (Sol Genomics Network: **http://solgenomics.net**, last accessed on 11 November 2019.) against the protein sequences of 130 DOXs from *Arabidopsis thaliana* (Kawai et al. 2014). The retrieved sequences had *E*-values $<1e^{-05}$ (**Supplementary Table S1**). Gene expression information for the extracted tomato *DOX*s was acquired from the transcriptome dataset of various tissues of *S. lycopersicum* cv. Heinz in the Tomato Functional Genomics Database (**http://ted.bti.cornell.edu/**, last accessed on 11 November 2019). We selected four genes (*Solyc02g062460*, *Solyc02g062500*, *Solyc03g095900* and *Solyc09g089580*) as α-tomatine 23-hydroxylase candidates that showed the highest ratios of RPKM in mature green (MG) fruits to 3-cm fruits (corresponding to immature green fruits) (**Supplementary Table S1**). Quantitative RT-PCR analysis revealed that the expression of the *Solyc02g062460* in MG fruits was higher than that in yellow fruits and that the transcript

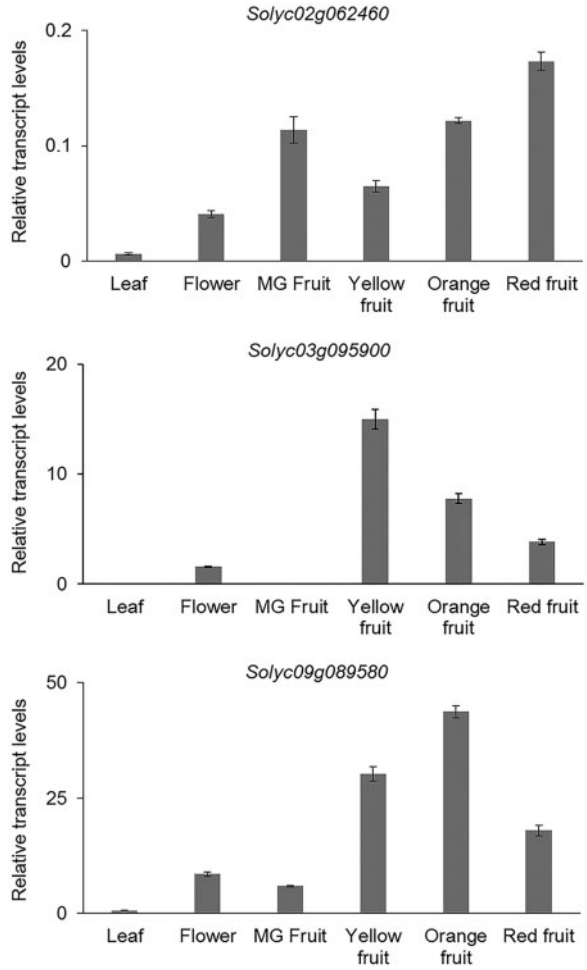

Fig. 2 Quantitative RT-PCR analysis of the expression patterns of candidate α-tomatine hydroxylase genes in various organs of tomato plants. Transcript levels of each gene are shown relative to that of *Ubiquitin* as an internal reference gene. Error bars indicate standard deviation ($n = 3$).

levels of *Solyc03g095900* and *Solyc09g089580* in MG fruits were lower than those in yellow fruits (**Fig. 2**). In contrast, *Solyc02g062500* expression was undetectable, and hence it was excluded from the candidate. The phylogenic classification of the plant DOX superfamily (Kawai et al. 2014) revealed that Solyc02g062460 is classified to clade DOXC20, which includes AOP1, AOP2 and AOP3, which are involved in glucosinolate metabolism in *A. thaliana* (Kliebenstein et al. 2001), whereas Solyc03g095900 and Solyc09g089580 are found in clade DOXC31, which includes AtGSL-OH, which is involved in glucosinolate biosynthesis in *A. thaliana* (Hansen et al. 2008).

In vitro assay using recombinant enzymes encoded by the candidate genes

To clarify the enzymatic activities of Solyc02g062460, Solyc03g095900 and Solyc09g089580, each recombinant protein was prepared by heterologous expression in *Escherichia coli*, but a soluble protein of Solyc03g095900 was not obtained. In vitro assays were performed with α-tomatine as a substrate, and the reaction mixtures were analyzed using liquid chromatography–mass spectrometry (LC–MS). Solyc09g089580 did not metabolize α-tomatine at all. In contrast, Solyc02g062460 produced a compound with a retention time of 8.2 min and a mass fragment ion at *m/z* 1050 (**Fig. 3A, B**). This product was identical to neorickiioside B in terms of both the retention time and the mass spectrum. Since the stereochemistry of neorickiioside B is different from a putative direct product of 23-hydroxylated α-tomatine, we purified the reaction product from the reaction mixture and used NMR to determine its chemical structure. The results confirmed the reaction product to be neorickiioside B (**Supplementary Table S2**). These results suggest that Solyc02g062460 catalyzes the C-23 hydroxylation of α-tomatine to form 23-hydroxytomatine and the product is subsequently isomerized to neorickiioside B. Thus, Solyc02g062460 was designated as Sl23DOX.

Next, we determined the substrate specificity of Sl23DOX to three SGAs (α-tomatine, α-solasonine and α-solanine), three steroidal saponins (dioscin, timosaponin A-III and protodioscin) and tomatidine (**Fig. 4**). Sl23DOX exhibited the highest activity to α-tomatine. The relative activities for solasonine, dioscin, tomatidine and timosaponin-III were 94%, 81%, 37% and 28% of that of α-tomatine, respectively. Sl23DOX did not metabolize α-solanine and protodioscin. These results suggest that spirosolane and spirostane glycosides harboring C-27 in the equatorial position are preferable substrates for Sl23DOX but solanidane and frostane glycosides are not metabolized.

SGA analysis in *Sl23DOX*-overexpressed transgenic tomato plants

To confirm the function of *Sl23DOX* in planta, we constructed transgenic tomato plants constitutively expressing *Sl23DOX* with a binary vector pKT263. Four *Sl23DOX* overexpression lines were obtained and two (#1 and #2 of pKT263) of them had remarkably higher Sl23DOX transcript levels than the non-transgenic control line (**Fig. 5A**). The leaves of the two lines accumulated neorickiioside B, which was not detected in leaves of the control (**Fig. 5B** and **Supplementary Fig. S1**), indicating that Sl23DOX converts α-tomatine to neorickiioside B in planta. Interestingly, the leaves of *Sl23DOX*-overexpressed tomato plants also accumulated lycoperoside C but not prosapogenin A and esculeoside A (**Supplementary Figs. S1, S2**), indicating that an acetyltransferase that converts neorickiioside B to lycoperoside C is present in the leaves of tomato plants whereas no lycoperoside C was detected in leaves of the control.

SGA analysis in *Sl23DOX*-silenced transgenic tomato plants

Next, to investigate the contribution of *Sl23DOX* to the metabolic conversion of α-tomatine to esculeoside A in tomato fruits, we generated transgenic tomato plants and silenced *Sl23DOX* expression with a binary vector, pKT264. Among eight *Sl23DOX*-silenced transgenic lines, fruits sampled 20 d after color turning in three independent lines (#2, #5 and #10; **Fig. 6**) had lower *Sl23DOX* transcript levels than the control (**Fig. 6A**) and accumulated α-tomatine, in contrast to the control (**Fig. 6B**). In addition, the fruits of these lines contained lower concentration of esculeoside A than the control (**Supplementary Fig. S3**). These results demonstrate that Sl23DOX is α-tomatine 23-hydroxylase and is involved in the

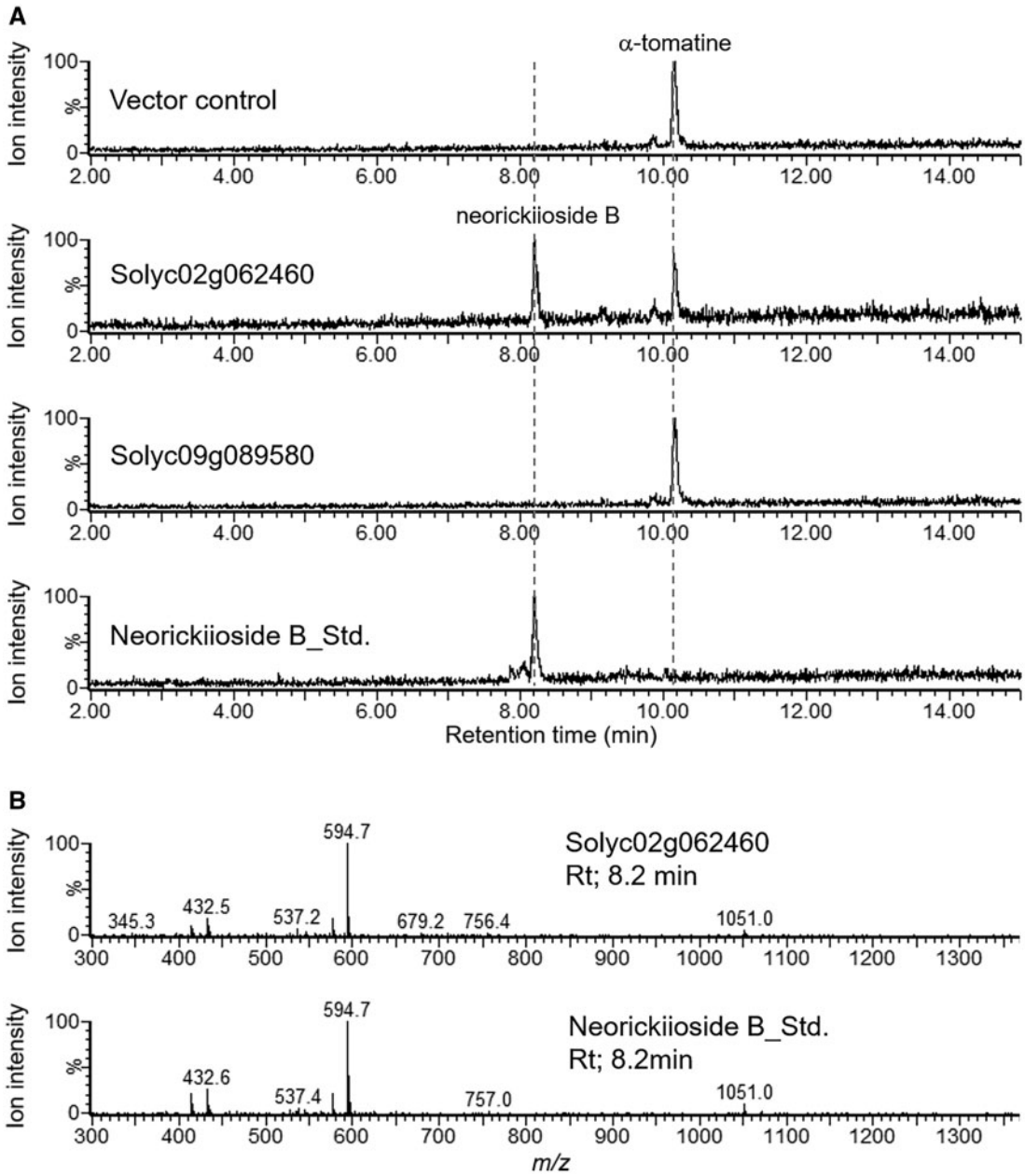

Fig. 3 LC–MS analysis of the reaction products from recombinant proteins of Solyc02g062460 and Solyc09g089580 with α-tomatine as a substrate. (A) Total ion chromatograms of the reaction products and the authentic compounds. (B) Mass spectra of the peaks shown in (A) at a retention time of 8.2 min.

metabolic conversion of α-tomatine to esculeoside A during tomato fruit ripening.

Discussion

Sl23DOX is a key enzyme for detoxifying α-tomatine and tomato domestication

α-Tomatine is a toxic and bitter compound, and therefore, tomato fruits containing reduced levels of α-tomatine have been selected during tomato domestication (Rick et al. 1994, Zhu et al. 2018). During the ripening process, α-tomatine is converted to esculeoside A (Iijima et al. 2013). The metabolic conversion is hypothesized to be subsequently modified via hydroxylation of α-tomatine at C-23, isomerization, acetylation at the C-23 hydroxy group, hydroxylation at C-27 and glycosylation at the C-27 hydroxy group (**Fig. 1**). In this study, in vitro enzyme assay using recombinant Sl23DOX protein and in vivo analyses of overexpression and silencing of the *Sl23DOX* gene in transgenic tomato plants clearly demonstrated that Sl23DOX catalyzes 23-hydroxylation of α-tomatine and the product is isomerized to neorickiioside B (**Fig. 3** and **Supplementary Table S2**). These results indicate that C-23 hydroxylation is the first step in the metabolic conversion of α-tomatine to esculeoside A and also that Sl23DOX is a key enzyme in the detoxification of α-tomatine during ripening. Thus, it is likely

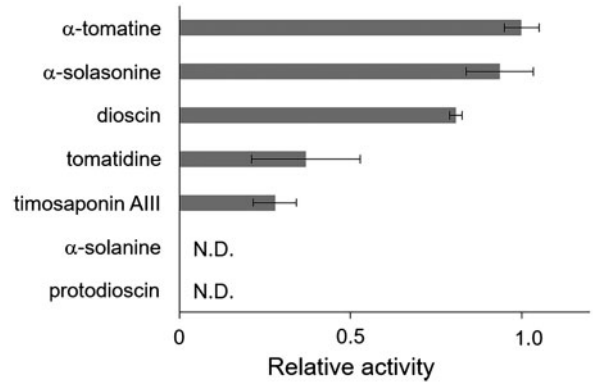

Fig. 4 Relative activities of recombinant Sl23DOX to several steroidal compounds. Error bars indicate standard deviation ($n = 3$). N.D., not detected.

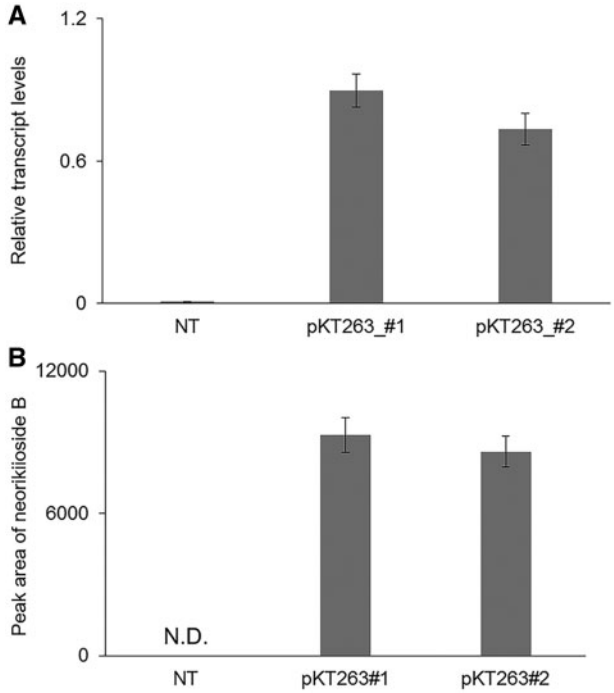

Fig. 5 Quantification of SGA contents in *23DOX*-overexpressed transgenic tomato plants. (A) Quantitative RT-PCR analysis of *23DOX* transcript levels in leaves of *23DOX*-overexpressed transgenic tomato plants. (B) Peak area of neorickiioside B contents in leaves of *23DOX*-overexpressed transgenic tomato plants. Error bars indicate standard deviation ($n = 3$). N.D., not detected; NT, non-transgenic control plants; pKT263#1 and pKT263#2, independent transgenic lines.

that *Sl23DOX* expression during fruit ripening was positively selected during tomato domestication. SGAs can also deter mutualists, such as birds, which are major seed dispersers, and, therefore, the detoxification of α-tomatine by *Sl23DOX* is important to tomato propagation.

Tomato is a member of a complex of 13 interfertile species that occupy a wide range of habitats in South America (Atherton and Rudich 1986). The most obvious domestication associated trait in tomato is a dramatic increase in fruit size, and this trait is controlled by a relatively small number of loci

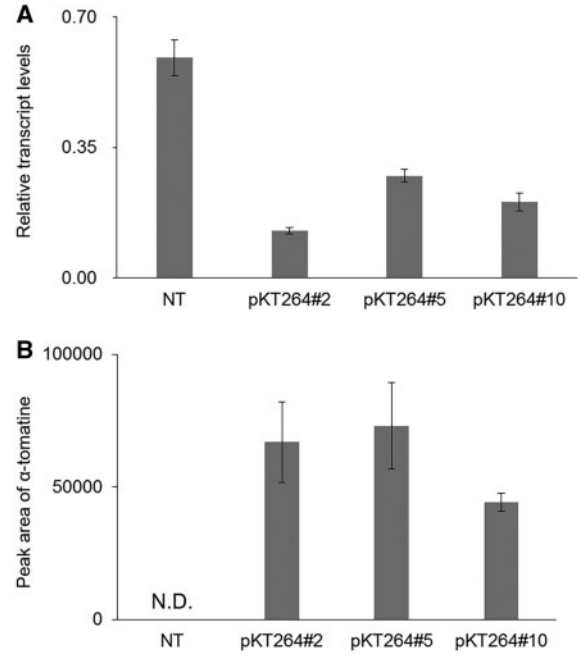

Fig. 6 Quantification of SGA contents in *23DOX*-silenced transgenic tomato plants. (A) Quantitative RT-PCR analysis of *23DOX* transcript levels in red fruits of *23DOX*-silenced transgenic tomato plants. (B) Peak area of α-tomatine in red fruits of *23DOX*-silenced transgenic tomato plants. Error bars indicate standard deviation ($n = 3$). N.D., not detected; NT, non-transgenic control plants; pKT264#2, pKT264#5 and pKT264#10, independent transgenic lines.

(Grandillo and Tanksley 1996). Genome-wide genetic diversity analyses showed that selections from wild tomato species *S. pimpinellifolium*, bearing small and red fruits, evolved into the semidomesticated *S. lycopersicum* var. *cerasiforme*, bearing fruits of small to medium weight, and that further domestication of *S. lycopersicum* var. *cerasiforme* gave rise to the cultivated tomato *S. lycopersicum* var. *lycopersicum*, bearing large fruits (Blanca et al. 2012). In *S. lycopersicum* var. *cerasiforme*, the bitter flavor accession LA2213 accumulates a high concentration of α-tomatine in the ripe fruit, whereas the sweet flavor accession LA2295 does not (Rick et al. 1994). In accordance with these findings, Iijima et al. (2013) confirmed that the fruit of LA2213 normally turn red after ripening, while the red fruit contained α-tomatine at a high level, in contrast to fruits of LA1090 and LA3911 of *S. lycopersicum* var. *lycopersicum*. These observations suggest that α-tomatine catabolism during fruit ripening of LA2213 might be suppressed by a mutation in the metabolic enzymes (e.g. Sl23DOX) or by a deterioration of a transport of α-tomatine from vacuole to the cytosol, where Sl23DOX is probably localized.

Sl23DOX promotes the configurational isomerization from C22β-N to the C22α-N

Our in vitro assay using recombinant Sl23DOX indicate that C-23 hydroxylation promotes configurational isomerization from C22β-N to C22α-N. We propose a model of the C-22 isomerization mechanism followed by C-23 hydroxylation (**Fig. 7**). In C-23 hydroxylation of α-tomatine at the axial

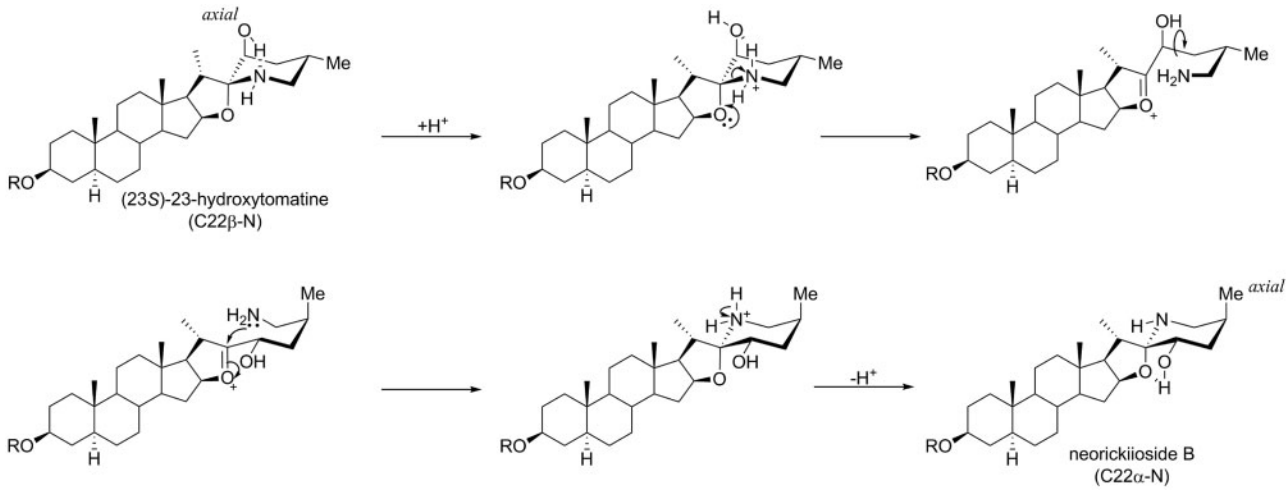

Fig. 7 Proposed mechanisms of spontaneous isomerization of (23*S*)-23-hydroxytomatine to neorickiioside B.

position (23*S*), the hydrogen atom of the C-23 hydroxy group forms a hydrogen bond with the nitrogen atom of the F-ring followed by the formation of cationic nitrogen and F-ring opening. In this situation, C-22 isomerization occurs and then F-ring closure forms neorickiioside B (C22α-N). As the 23-hydroxy group of neorickiioside B (C22α-N) is located in the proximity of the C-16 ether oxygen with C-22, a hydrogen bond can form between the 16-oxygen atom and the hydrogen atom of the 23-hydroxy group. For this reason, the configuration is more stable than (23*S*)-23-hydroxytomatine (C22β-N). In contrast, if α-tomatine is hydroxylated at C-23 in the equatorial position (23*R*) to form (23*R*)-23-hydroxytomatine, the 23-hydroxy group is distantly located from the nitrogen atom of the F-ring, which results in the retention of the configuration (C22β-N). Iijima et al. (2013) reported that all SGAs, except for habrochaitoside A, that were isolated and structurally determined from eight tomato accessions have the C22α-N configuration in contrast to that of α-tomatine but the same as esculeoside A. In addition, Schwahn et al. (2014) showed that the contents of esculeoside A and deacetyl-esculeoside A were significantly higher than those of lycoperoside F (C22β-N and 23*R* configurational stereoisomer of esculeoside A) and diacetyl-lycoperoside F in the mature fruits of four tomato accessions, LA3475 (*S. lycopersicum* cv. M82), LA1589 (*S. pimpinellifolium*), LA1274 (*Solanum peruvianum*) and LA2133 (*Solanum neorickii*). These findings suggest that Sl23DOX and the orthologs from other accessions specifically or preferentially catalyze 23-hydroxylation at the 23*S* configuration in the fruits of these accessions and LA3911 (*S. lycopersicum* cv. Micro-Tom). Furthermore, the minimal amounts of C22β-N-type SGAs in these accessions would imply minor (23*R*)-23-hydroxylation activity of Sl23DOX or the existence of another DOX that catalyzes hydroxylation in the 23*R* configuration.

Materials and Methods

Chemicals

Authentic samples of α-solanine and tomatidine hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dioscin and protodioscin were purchased from Carbosynth (Berkshire, UK). α-Tomatine, α-solasonine and timosaponin A-III were purchased from Tokyo Kasei (Tokyo, Japan), AvaChem Scientific (San Antonio, TX, USA) and Fujifirm Wako Pure Chemical (Osaka, Japan), respectively. Neorickiioside B and lycoperoside C were isolated as described previously (Iijima et al. 2013).

Heterologous expression of recombinant proteins in *E. coli*

The cDNA fragments containing open reading frame of *Sl23DOX*, *Solyc03g095900* and *Solyc09g089580* from LA3911 (*S. lycopersicum* cv. Micro-Tom) were amplified by PCR with the primer sets 1 and 2, 3 and 4 and 5 and 6, respectively (**Supplementary Table S3**), using three cDNA clones as template, LEFL2019P01, LEFL2027P04 and LEFL2046F14, respectively, which were provided by the National BioResource Project (NBRP) Tomato (AMED, Japan). The amplified DNA fragments were ligated into pMD20 (TaKaRa, Shiga Japan), digested with each restriction site and ligated into the corresponding sites of pGEX4T-1 (GE healthcare, Buckinghamshire, England, UK). Heterologous expression of recombinant proteins in *E. coli* strain BL21 (DE3) (TMerck Millipore, Burlington, MA, USA) transformed with the constructed plasmids was performed, and the obtained soluble proteins were purified using spin columns as previously described (Nakayasu et al. 2017). The concentration of the purified proteins was determined using the Bradford system. The purified recombinant proteins were visualized by SDS–PAGE. The proteins were revealed by staining the gel using Coomassie brilliant blue R-250.

In vitro enzyme activity assay

The reaction mixture (100 µl) consisted of 100 mM Bistris–HCl (pH 7.2), 5 mM 2-ketoglutaric acid, 10 mM sodium ascorbate, 0.2 mM $FeSO_4$, 100 µM α-tomatine as substrate and the purified recombinant protein as enzyme. The reaction was initiated by the addition of enzyme, carried out at 30°C for 1 h and stopped by boiling for 5 min. The reaction product was diluted with an equal volume of methanol and filtrated using a polytetrafluoroethylene filter (Waters, Milford, MA, USA). LC–MS analysis of the reaction product was performed as previously described (Abdelkareem et al. 2017) with the following minor modifications: the mobile phases were water with 0.1% (v/v) formic acid (A) and acetonitrile (B), using a gradient elution of 10–40% B at 0–15 min (linear gradient); mass spectra were obtained by MS scan mode with a mass range of *m*/*z* 250–1400.

Identification of the reaction product catalyzed by Sl23DOX

To determine the structure of the product of the enzymatic reaction catalyzed by recombinant Sl23DOX, the enzymatic reaction was performed using 30 ml of reaction mixture as described above with a minor modification; the reaction was conducted using the crude enzyme overnight. The reaction mixture was loaded to Sep-Pak Plus C18 Cartridges (Waters) followed by a stepwise elution of H_2O/

MeOH (90%:10%, 65%:35%, 50%:50%, 25%:75% and 0%:100%, 5 ml in each step) to yield stepwise fractions. Each fraction was analyzed by LC–MS as described above, and then, a 75% MeOH fraction including the Sl23DOX reaction product was subjected to preparative scale HPLC. The eluents were collected at 0–5, 5–10, 10–15 and 15–20 min. Each fraction was analyzed by LC–MS as described above, and then, the eluent at 10–15 min containing the 23DOX product was concentrated in vacuo and the dried residue was dissolved in pyridine-d_5 (C_5D_5N; Sigma-Aldrich). NMR spectra were recorded using a Bruker AVANCEIII 600 spectrometer (Bruker BioSpin Corp., Billerica, MA, USA) at 600 MHz for ^{1}H and 151 MHz for ^{13}C. The NMR chemical shifts were referenced to tetramethylsilane. The ^{13}C NMR spectroscopic data are shown in **Supplementary Table S2**.

Biochemical analysis of recombinant Sl23DOX

We determined the substrate specificity of recombinant Sl23DOX to several steroidal compounds. The activity for each substrate was assayed using α-tomatine, α-solasonine, dioscin, timosaponin A-III, tomatidine, α-solanine and protodioscin at 100 μM. The reaction and LC–MS analysis of the reaction products were performed as described above. Sl23DOX metabolized α-tomatine, α-solasonine, dioscin, timosaponin A-III and tomatidine but not α-solanine and protodioscin, and then, the five former substrates were used for the determination of the conversion ratio of Sl23DOX to vector control for each substrate. The ratios were calculated from peak area of the residual substrates in reaction mixture that were measured by selected ion recording modes with *m*/*z* 1035.0, 885.1, 869.9, 741.9 and 416.6 for α-tomatine, α-solasonine, dioscin, timosaponin A-III and tomatidine, respectively.

Generation of transformation vectors, plant transformation and growth conditions

A binary vector pKT263 for *Sl23DOX* overexpression was constructed from the binary vector pKT11 (Umemoto et al. 2001) by locating a sequence including the coding region of *Sl23DOX* under the control of cauliflower mosaic virus 35S (CaMV35S) promoter in the T-DNA region. An RNAi binary vector pKT264 for *Sl23DOX* silencing was constructed from the binary vector pKT11 by locating two 322-bp fragments of *Sl23DOX* in opposite interposing the third intron of *A. thaliana* gene *At4g14210* under CaMV35S promoter as previously described (Umemoto et al. 2016). The 322-bp fragment of *Sl23DOX* cDNA, as described above, was PCR amplified using primers 7 and 8 containing restriction sites (**Supplementary Table S3**). The binary vectors pKT263 and pKT264 were electroporated into *Agrobacterium tumefaciens* GV3101 MP90. LA3911 (*S. lycopersicum* cv. Micro-Tom) was provided by NBRP Tomato and was transformed using GV3101 MP90 cells with pKT263 or pKT264 as previously reported (Sun et al. 2006). In vitro-grown plants were cultured at 25°C under a 16-h light/8-h dark cycle. Transformants were individually selected by genomic PCR of the shoots with primers 9 and 10 (**Supplementary Table S3**; Sawai et al. 2014) targeting the kanamycin resistance gene on the T-DNA region integrated into the tomato genome. Total RNA was prepared from leaves of in vitro-cultured plants using the RNeasy plant mini-kit (QIAGEN, Hilden, Germany) and RNase-Free DNase Set (QIAGEN). The extracted total RNA was used to synthesize the first-strand cDNA using a Transcriptor First Strand cDNA Synthesis Kit (TOYOBO, Osaka, Japan). *Sl23DOX* transcript levels in the transformants obtained were measured as described in the following section.

Quantitative RT-PCR analysis

Quantitative RT-PCR analysis was conducted with LightCycler®Nano (Roche, Basel, Switzerland) using THUNDERBIRDTM SYBR® qPCR Mix (TOYOBO). Quantitative RT-PCR for α-tomatine C23-hydroxylase candidate genes in various tissues of tomato was performed with the following primers sets: 11 and 12 for *Solyc02g062460* (*Sl23DOX*), 13 and 14 for *Solyc02g062500*, 15 and 16 for *Solyc03g095900* and 17 and 18 for *Solyc09g089580* (**Supplementary Table S3**), using cDNA from leaves, flowers, MG, yellow, orange and red fruits of LA3911 (*S. lycopersicum* cv Micro-Tom) that were prepared as described by Nakayasu et al. (2017). Quantitative RT-PCR for *Sl23DOX* in *Sl23DOX*-overexpressing and *Sl23DOX*-silenced transgenic tomato plants was performed with primers 19 and 20, using cDNA from the leaves as template. Primers 21 and 22 (**Supplementary Table S3**; Nakayasu et al. 2017), which targeted the *Ubiquitin* gene, were used as a control. Cycling was undertaken for 10 min at 95°C, 45 cycles of 10 s at 95°C, 10 s at 59°C and 15 s at 72°C for amplification, followed by holding for 30 s at 95°C and ramping up from 60 to 95°C at 0.1°C/s for melting curve analysis. Three biological repeats were analyzed in duplicate. The genes' expression levels were normalized against the values obtained for *Ubiquitin*, which was used as an internal reference in tomato. Data acquisition and analysis were performed using LightCycler®Nano software (Roche).

LC–MS analysis of SGAs in *Sl23DOX*-overexpressing and *Sl23DOX*-silenced transgenic plants

Extraction of SGAs contained in *Sl23DOX*-overexpressing and *Sl23DOX*-silenced transgenic plants were performed as previously described (Iijima et al. 2009). LC–MS/MS analysis was conducted on a system composed of an Acquity UPLC system (Waters) and an Acquity quadruple tandem mass spectrometer (Waters) Data acquisition and analysis were performed using MassLynx 4.1 software (Waters). Extracts (2 μl) were separated through an Acquity UPLC HSS T3 (1.8 μm, 2.1 × 100 mm, Waters). The column temperature was set at 30°C and the flow rate at 0.2 ml/min. The mobile phases were 20% methanol (A) and 100% methanol (B), using a gradient elution of 0% B at 0–3 min, 0–30% B at 3–5 min, 30–50% B at 5–10 min, 50–100% B at 10–25 min. SGAs were detected by multiple reaction monitoring under the following conditions: transition, *m*/*z* 1034.5 > 416.4, > 435.4, and > 1016.4, and 1056.5 > 799.4 for α-tomatine, *m*/*z* 1050.5 > 255.2, > 414.4, > 432.4, and > 1032.4 for neorickiioside B, *m*/*z* 1092.5 > 255.4, > 414.4, > 576.4, > 1014.4, and > 1032.4 for lycoperosde C, and *m*/*z* 1270.6 > 163.2, > 545.2, and > 1210.5 for esculeoside A; cone voltage, 60 V for α-tomatine and neorickiioside B, 65 V for lycoperosde C, and 80 V for esculeoside A; collision energy, 50 eV with exception of transition *m*/*z* 1034.5 > 416.4 for α-tomatine that was set at 40 eV.

Supplementary Data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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