The Plant Cell, Vol. 26: 3763–3774, September 2014, www.plantcell.org © 2014 American Society of Plant Biologists. All rights reserved.

Sterol Side Chain Reductase 2 Is a Key Enzyme in the Biosynthesis of Cholesterol, the Common Precursor of Toxic Steroidal Glycoalkaloids in Potato[W][OPEN]

Satoru Sawai,[a,b,1] Kiyoshi Ohyama,[a,c,1] Shuhei Yasumoto,[d] Hikaru Seki,[a,d] Tetsushi Sakuma,[e] Takashi Yamamoto,[e] Yumiko Takebayashi,[a] Mikiko Kojima,[a] Hitoshi Sakakibara,[a] Toshio Aoki,[f] Toshiya Muranaka,[a,d] Kazuki Saito,[a,b,2] and Naoyuki Umemoto[g]

[a] RIKEN Center for Sustainable Resource Science, Tsurumi-ku, Yokohama, Kanagawa 230-0045, Japan
[b] Graduate School of Pharmaceutical Sciences, Chiba University, Chuo-ku, Chiba 260-8675, Japan
[c] Department of Chemistry and Materials Science, Tokyo Institute of Technology, Meguro-ku, Tokyo 152-8551, Japan
[d] Department of Biotechnology, Graduate School of Engineering, Osaka University, Suita, Osaka 565-0871, Japan
[e] Department of Mathematical and Life Sciences, Graduate School of Science, Hiroshima University, Higashi-Hiroshima, Hiroshima 739-8526, Japan
[f] Department of Applied Biological Sciences, Nihon University, Fujisawa, Kanagawa 252-0880, Japan
[g] Central Laboratories for Key Technologies, Kirin Co., Kanazawa-ku, Yokohama, Kanagawa 236-0004, Japan

Downloaded from https://academic.oup.com/plcell/article/26/9/3763/6100345 by U.S. Department of Justice user on 16 August 2022

Potatoes (*Solanum tuberosum*) contain α-solanine and α-chaconine, two well-known toxic steroidal glycoalkaloids (SGAs). Sprouts and green tubers accumulate especially high levels of SGAs. Although SGAs were proposed to be biosynthesized from cholesterol, the biosynthetic pathway for plant cholesterol is poorly understood. Here, we identify sterol side chain reductase 2 (SSR2) from potato as a key enzyme in the biosynthesis of cholesterol and related SGAs. Using in vitro enzyme activity assays, we determined that potato SSR2 (St SSR2) reduces desmosterol and cycloartenol to cholesterol and cycloartanol, respectively. These reduction steps are branch points in the biosynthetic pathways between C-24 alkylsterols and cholesterol in potato. Similar enzymatic results were also obtained from tomato SSR2. St *SSR2*-silenced potatoes or St *SSR2*-disrupted potato generated by targeted genome editing had significantly lower levels of cholesterol and SGAs without affecting plant growth. Our results suggest that St *SSR2* is a promising target gene for breeding potatoes with low SGA levels.

INTRODUCTION

Steroidal glycoalkaloids (SGAs) are famous as toxic compounds in solanaceous plants that include the world's major food crops such as potato (*Solanum tuberosum*) and tomato (*Solanum lycopersicum*) (Friedman, 2002, 2006). α-Chaconine and α-solanine account for most of the SGAs in cultivated potato and are especially abundant in sprouts, the green peel of tubers, and other aerial parts. To avoid postharvest increases in SGAs, harvested potatoes must be stored in the dark at low temperature (Kozukue and Mizuno, 1990). In potato breeding, introducing wild potato species as a genetic resource could increase the SGA level (Jacobsen and Rousselle, 1993). Thus, reducing the SGA level is an important target for potato breeding.

Feeding studies with isotope-labeled substrates suggested that SGAs are biosynthesized via cholesterol as a common precursor (Figure 1) (Tschesche and Hulpke, 1966, 1967; Heftmann et al., 1967). Cholesterol is converted to SGAs by a series of modifications that are predicted to involve oxidation, transamination and glycosylation (Ohyama et al., 2013). The recent releases of the potato (Xu et al., 2011) and tomato (Tomato Genome Consortium, 2012) genome sequences have accelerated the identification of genes encoding these enzymes in the SGA biosynthetic pathway (Moehs et al., 1997; McCue et al., 2005, 2006, 2007; Itkin et al., 2011, 2013). Modifying the SGA biosynthetic pathway by targeting potato genes encoding the oxidases or glycosyltransferases results in decreased levels of SGAs; however, the decrease in SGAs is associated with an increase in levels of intermediates between cholesterol and SGAs or unexpected derivatives of the intermediates (McCue et al., 2005, 2006, 2007; Itkin et al., 2011, 2013). This unintended metabolic change may pose a risk to potato consumers. As a more ideal way of engineering potatoes, reducing the supply of cholesterol, a common precursor for SGA biosynthesis, should result in lower levels of SGAs without a corresponding increase in levels of undesired intermediates between cholesterol and SGAs or their derivatives. However, the biosynthetic mechanism for cholesterol in plants has not been fully elucidated.

Generally, plants produce C-24 alkylsterols including campesterol, a precursor of the phytohormone brassinolide (Figure 1). The primary structural difference between the C-24 alkylsterols and cholesterol is the presence of an alkyl group at the C-24 position of the side chain (Benveniste, 1986). The C-24 alkylsterols have a methyl or an ethyl group at C-24 resulting from the addition of one

[1] These authors contributed equally to this work.
[2] Address correspondence to kazuki.saito@riken.jp.
The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Kazuki Saito (kazuki.saito@riken.jp).
[W] Online version contains Web-only data.
[OPEN] Articles can be viewed online without a subscription.
www.plantcell.org/cgi/doi/10.1105/tpc.114.130096

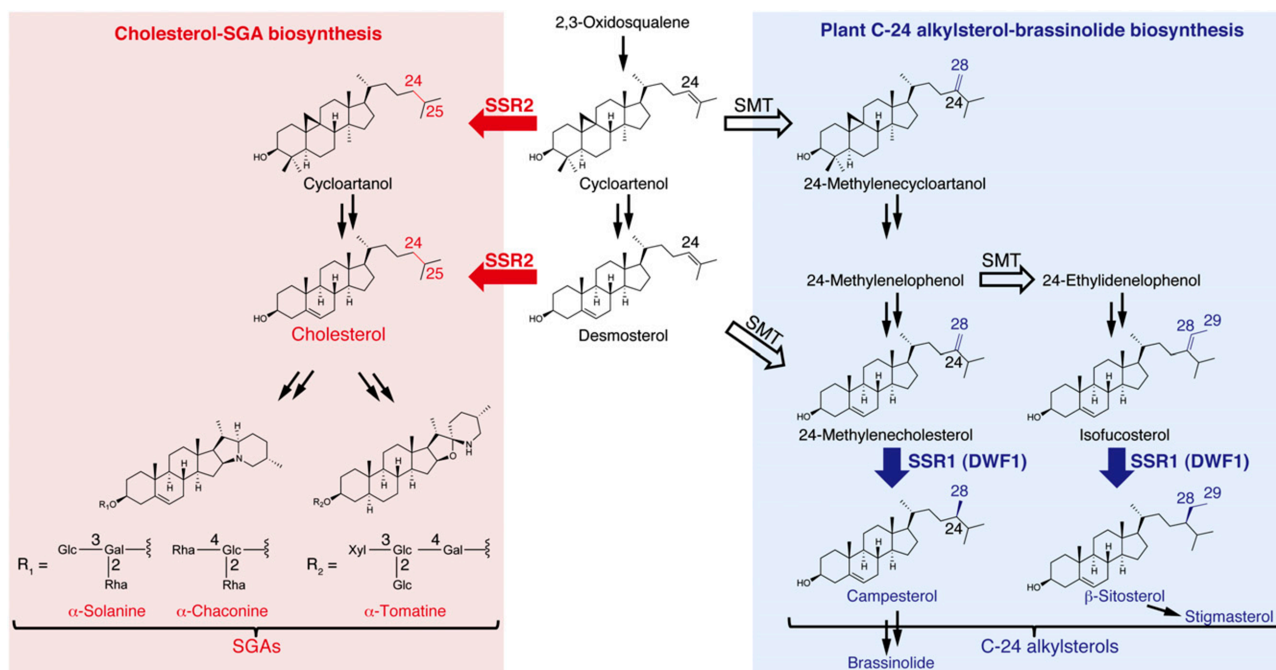

Figure 1. Biosynthetic Pathways of Steroidal Glycoalkaloids and Plant C-24 Alkylsterols.

Plant C-24 alkylsterols and cholesterol, the common intermediates in steroidal glycoalkaloid biosynthesis, are biosynthesized from cycloartenol. Red arrows indicate reduction steps mediated by SSR2. Blue arrows indicate reduction steps mediated by SSR1. Open black arrows indicate transmethylation steps mediated by SMTs.

or two methyl groups to $\Delta^{24(25)}$-sterols, such as cycloartenol and desmosterol catalyzed by sterol methyltransferases (SMTs) (Husselstein et al.,1996; Shi et al., 1996; Bouvier-Navé et al., 1997; Grebenok et al., 1997). Diminuto/Dwarf1 (DWF1) (Klahre et al., 1998; Choe et al., 1999) of *Arabidopsis thaliana* has been proposed to reduce alkylidene groups generated by SMT reactions to alkyl groups (Figure 1). The *dwf1* mutant has a dwarf phenotype due to the lack of brassinosteroids (Kauschmann, et al., 1996). In the cholesterol biosynthetic pathway of animals including humans, the C-24 position of $\Delta^{24(25)}$-sterols, such as desmosterol, is reduced by 24-dehydrocholesterol reductase (DHCR24) (Waterham et al., 2001), a homolog of DWF1. Although cholesterol is generally present only in trace amounts in plants, solanaceous plants accumulate considerable amounts of SGAs derived from cholesterol. To meet this high demand for cholesterol, these plants may have a cholesterol biosynthetic mechanism distinct from that for ordinary plant sterol biosynthesis. In this study, a gene encoding sterol side chain reductase 2 (SSR2) committed to cholesterol biosynthesis in plants was identified and used to engineer potatoes with lower levels of SGAs.

RESULTS

Potato and Tomato Have Two Types of Genes Encoding DHCR24 Homologs

Using the human DHCR24 amino acid sequence as a query, BLAST (W. Gish, http://blast.wustl.edu and http://www.advbiocomp.com/blast/obsolete; Altschul et al., 1997) analysis against potato unigene databases from the Dana-Farber Cancer Institute Potato Gene Index (Quackenbush et al., 2001), and the Sol Genomics Network (Bombarely et al., 2011) allowed us to select two types of cDNA sequences designated as St *SSR1* and St *SSR2*. In contrast, *DWF1* is a single copy gene in *Arabidopsis*. We also found two full-length cDNA sequences (Sl *SSR1* and Sl *SSR2*) similar to *DHCR24* from a tomato unigene database from the Sol Genomics Network. The nucleotide sequence identities between St *SSR1* and St *SSR2* and between Sl *SSR1* and Sl *SSR2* are 81 and 79%, respectively. Phylogenetic analysis of the coding sequences of *DHCR24* homologous genes from plants (Figure 2; Supplemental Data Set 1) indicates that *SSR1* and *SSR2* are paralogs in the *Solanum* genus. Although genes associated with SGA biosynthesis in potato and tomato were reported to be clustered on chromosomes 7 and 12 (Itkin et al., 2013), *SSR* genes in potato and tomato are positioned on chromosome 2 and are more than 10 Mbp away from each other (St *SSR1*, PGSC0003DMG400011801, and St *SSR2*, PGSC0003DMG400021142, in Xu et al., 2011; Sl *SSR1*, Solyc02g030170.2, and Sl *SSR2*, Solyc02g069490.2, in Tomato Genome Consortium, 2012). All four deduced proteins were predicted to have a flavin adenine dinucleotide binding domain and a transmembrane region by the PROSITE (Sigrist et al., 2002) and SOSUI (Hirokawa et al., 1998) programs, respectively (Supplemental Data Set 2).

In Vivo Enzymatic Activity Assays of SSRs

We presumed that one SSR would catalyze the reduction of $\Delta^{24(25)}$-sterol involved in cholesterol biosynthesis and the other

Downloaded from https://academic.oup.com/plcell/article/26/9/3763/6100345 by U.S. Department of Justice user on 16 August 2022

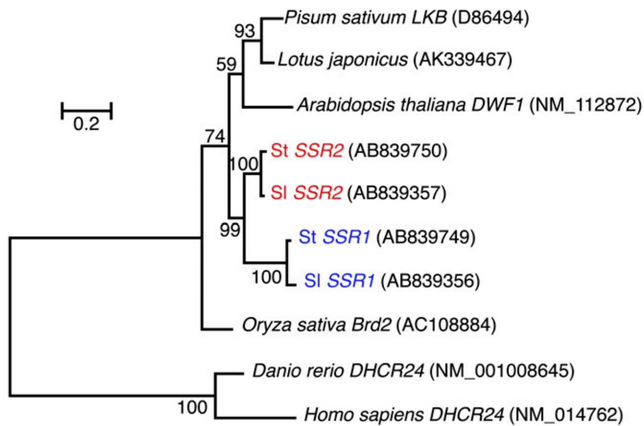

Figure 2. Phylogenetic Relationships among *DHCR24* Homologous Genes from Plants.

*DHCR24*s of *Homo sapiens* and *Danio rerio* were used as outgroups. Numbers beside the nodes indicate bootstrap values (in percentages of 1000 replicates). The scale bar indicates the nucleotide substitution ratio. GenBank/EMBL/DDBJ accession numbers for the nucleotide sequences of the genes are given in parentheses.

SSR would mediate reduction of the $\Delta^{24(28)}$-double bond of C-24 alkylsterol biosynthetic intermediates. To investigate the reduction activities of the four putative SSRs from potato and tomato, the genes were expressed in yeast strains T31 and T21 that have modified ergosterol biosynthesis (Figure 3A; Supplemental Figure 1). The T31 strain accumulates a $\Delta^{24(25)}$-sterol, desmosterol, and the T21 strain accumulates a precursor of campesterol, 24-methylenecholesterol. Ethyl acetate extracts of the yeast cells were analyzed by gas chromatography-mass spectrometry (GC-MS) and compared with standard samples (Supplemental Figure 2). The T31 cells expressing St *SSR2* and Sl *SSR2* accumulated remarkable amounts of cholesterol compared with cells expressing St *SSR1* and Sl *SSR1* (Figure 3B). When T21 served as the host strain, the cells expressing St *SSR1* and Sl *SSR1* accumulated marked amounts of campesterol compared with the cells expressing St *SSR2* and Sl *SSR2*. The T21 cells expressing St *SSR2* and Sl *SSR2* also accumulated cholesterol (Figure 3C). In the T21 cells, 24-methylenecholesterol is thought to be biosynthesized via $\Delta^{24(25)}$-sterols. The accumulation of cholesterol in the T21 cells expressing St *SSR2* and Sl *SSR2* may be attributed to the reduction of the $\Delta^{24(25)}$-sterols before C-24 methylation of the $\Delta^{24(25)}$-sterols catalyzed by ERG6 (yeast Δ^{24}-sterol methyltransferase). These results indicate that SSR2 and SSR1 have different substrate specificities. St *SSR2* and Sl *SSR2* encode the sterol $\Delta^{24(25)}$ reductases. In contrast, St *SSR1* and Sl *SSR1* encode the sterol $\Delta^{24(28)}$ reductases. Thus, St *SSR1* and Sl *SSR1* are orthologous genes of *DWF1* in potato and tomato, respectively.

In Vitro Enzymatic Activity Assays of SSRs

The in vivo cholesterol production by the T31 and T21 yeasts expressing SSR2 suggests that St SSR2 may be able to reduce a variety of the $\Delta^{24(25)}$-sterols, including lanosterol, the first $\Delta^{24(25)}$-sterol in yeast, and desmosterol (Supplemental Figure 1). In plants, cycloartenol is the first $\Delta^{24(25)}$-sterol. If cycloartenol is reduced to cycloartanol by SSR2, the biosynthetic pathway would be efficiently redirected toward cholesterol (Figure 1) (Diener et al., 2000; Schaeffer et al., 2000; Sitbon and Jonsson 2001; Holmberg et al., 2002; Arnqvist et al., 2003). We conducted in vitro assays to examine whether SSR2 has cycloartenol reductase activity and to compare the substrate specificities of SSR1 and SSR2 (Figure 4). St *SSR1*, St *SSR2*, Sl *SSR1*, and Sl *SSR2* were heterologously expressed in an *erg4* [deficient in sterol $\Delta^{24(28)}$ reductase gene] yeast strain to avoid contamination of the endogenous sterol $\Delta^{24(28)}$ reductase activity into the in vitro SSR enzymatic assays. Homogenates of the *erg4* yeast cells expressing St *SSR1*, St *SSR2*, Sl *SSR1*, or Sl *SSR2* were incubated with cycloartenol, desmosterol, or 24-methylenecholesterol, and the *n*-hexane extracts of the incubated samples were analyzed by GC-MS. St SSR2 and Sl SSR2 efficiently converted cycloartenol to the $\Delta^{24(25)}$ reduction product, cycloartanol, and the reaction was NADPH dependent (Figure 4B). St SSR2 and Sl SSR2 also converted desmosterol to cholesterol in an NADPH-dependent manner (Figure 4C), a result consistent with the in vivo assay that indicated the production of cholesterol in yeast cells expressing *SSR2* (Figure 3B). On the other hand, St SSR1 and Sl SSR1 efficiently reduced 24-methylenecholesterol to campesterol. The weak activity was also shown by St SSR2 and

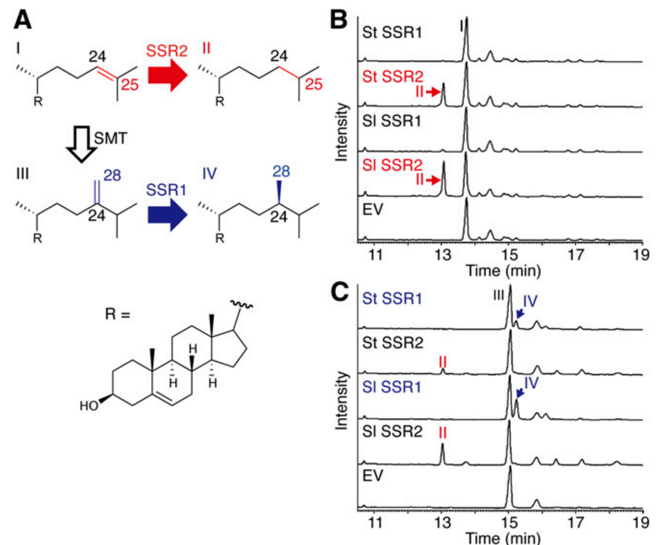

Figure 3. In Vivo Production of Cholesterol or Campesterol in Yeast Cells Transformed with *SSR*s.

(A) Reduction of sterol side chains by SSRs from potato and tomato to produce cholesterol (II) or campesterol (IV) in yeasts producing desmosterol (I) or 24-methylencholesterol (III), respectively. Red arrows indicate reduction steps mediated by SSR2. Blue arrows indicate reduction steps mediated by SSR1. The open black arrow indicates a Δ^{24}-methylation step mediated by SMT.

(B) and **(C)** Analysis of the extracts of *SSR*-expressing yeast cells by GC-MS (total ion chromatograms). *SSR*s from potato and tomato were functionally expressed in desmosterol-producing yeast **(B)** and 24-methylenecholesterol-producing yeast **(C)**. EV, empty vector control. Peak labels refer to **(A)**.

Downloaded from https://academic.oup.com/plcell/article/26/9/3763/6100345 by U.S. Department of Justice user on 16 August 2022

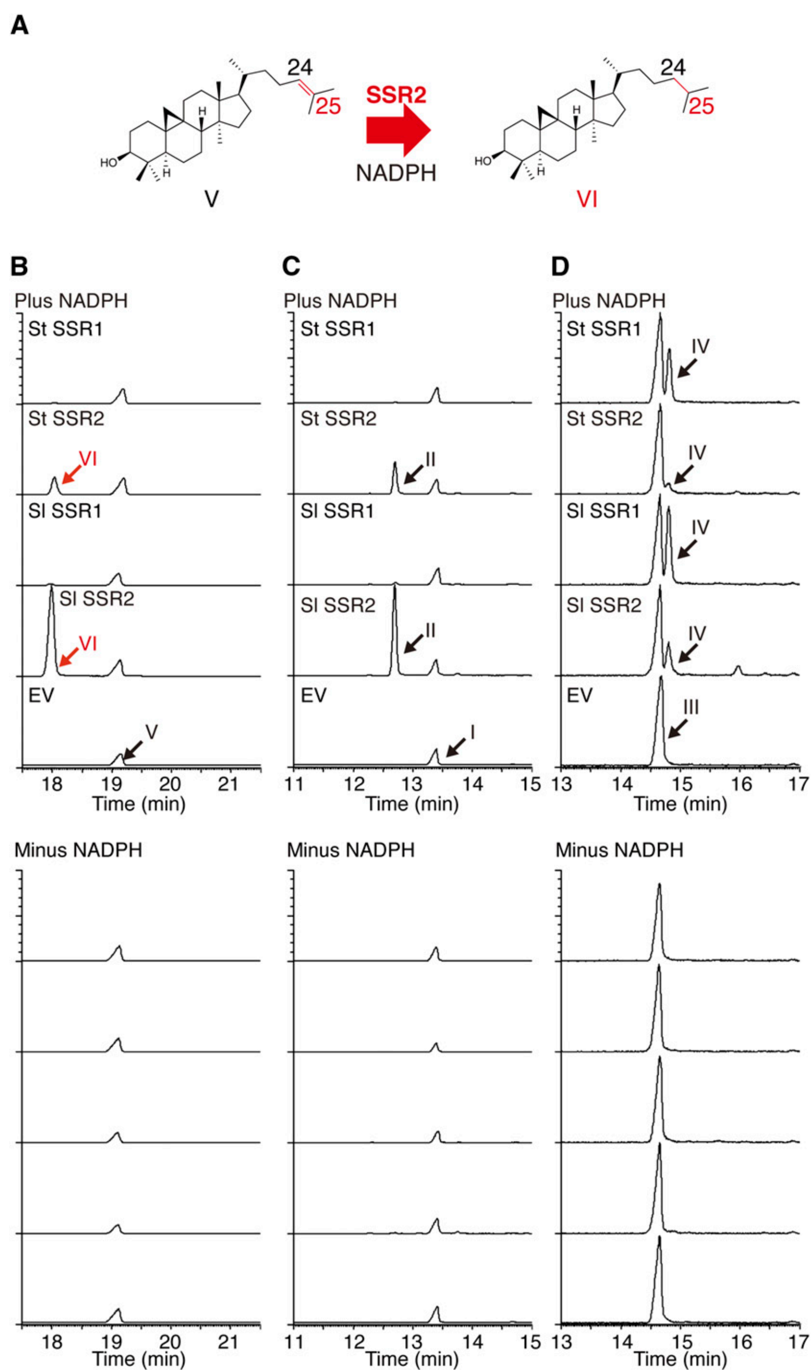

Figure 4. In Vitro Enzymatic Assays of SSRs.

(A) SSR2 activity for the $\Delta^{24(25)}$ reduction of cycloartenol (V) to cycloartanol (VI).

(B) to **(D)** In vitro assays using homogenates prepared from *erg4* yeast cells expressing tomato or potato *SSR*s with cycloartenol **(B)**, desmosterol **(C)**, and 24-methylenecholesterol **(D)** as the substrates. The assays were performed in the absence (minus NADPH) or presence of NADPH (plus NADPH). The reaction products were analyzed by GC-MS. Traces are extracted ion chromatograms at *m/z* 395, 368 and 382, respectively. EV, empty vector control. Peak labels refer to Figures 3A and 4A.

Downloaded from https://academic.oup.com/plcell/article/26/9/3763/6100345 by U.S. Department of Justice user on 16 August 2022

Sl SSR2 (Figure 4D). St SSR1 and Sl SSR1 also have very weak reductase activity toward cycloartenol and desmosterol (Supplemental Figure 3). These results indicate again that SSR1 and SSR2 have different substrate specificities. In addition, the $\Delta^{24(25)}$ reduction activity against cycloartenol of SSR2 suggests that SSR2 effectively directs the sterol biosynthetic flow toward the cholesterol-SGA pathway in potato and tomato.

Analyses of *SSR2*-Silenced Potato Plants

To verify the contribution of St *SSR2* in the biosyntheses of cholesterol and SGAs in potato plants, we constructed St *SSR2*-silenced potato plants by transformation with an RNA interference (RNAi) vector (Supplemental Figure 4A). The RNAi vector was constructed from a 269-bp fragment of St *SSR2* cDNA that did not match over 21 consecutive nucleotides with St *SSR1*. Four independent St *SSR2*-RNAi potato plant lines (#2, #10, #11, and #12 in Figures 5A to 5D) were identified that had remarkably reduced St *SSR2* transcript levels (Supplemental Figure 4B). In these selected RNAi lines, the levels of predominant SGAs (α-solanine and α-chaconine) in the stems of in vitro-grown plants (Figure 5A) and tuber peels (Figure 5B) were significantly lower by 10% or more compared with the nontransformant. The cholesterol content was also significantly lower in the St *SSR2*-RNAi lines (Figure 5C). The levels of campesterol and stigmasterol were unchanged despite the decrease in β-sitosterol content (Figure 5C). The *dwf1* mutant is well known for a dwarfed phenotype caused by a deficiency in brassinosteroids (Kauschmann, et al., 1996); however, the four St *SSR2*-RNAi lines showed normal growth and harvest yield (Figure 5D; Supplemental Figure 4C). We also analyzed levels of the major biologically active brassinosteroids, brassinolide and castasterone (Kim et al., 2005; Nomura et al., 2005), in two St *SSR2*-RNAi lines and the nontransformant. The levels of castasterone, which were under one thousandth of the cholesterol level in the nontransformant, were similar in the two transformants, and brassinolide levels were too low to quantify (Supplemental Table 1). These results indicate that St *SSR2* is devoted to the production of cholesterol and SGAs in potato plants. The data also suggest that St *SSR1* is responsible for C-24 alkylsterols and brassinolide biosyntheses. Levels of C-24 alkylsterol intermediates, such as 14α-methylcholesta-8,24-dien-3β-ol, 4α,14α-dimethylcholesta-8,24-dien-3β-ol, and isofucosterol, in the St *SSR2*-silenced lines were higher relative to those of the non-transformant (Supplemental Figures 4D and 4E). Similar results were also obtained from Sl *SSR2*-silenced tomatoes (Supplemental Figure 5).

Generation of an St *SSR2*-Disrupted Tetraploid Potato by Targeted Genome Editing

We also constructed an St *SSR2*-disrupted potato plant by transformation with a vector bearing a pair of transcription activator-like effector nuclease (TALEN) (Mahfouz et al., 2011; Cermak et al., 2011) constructs targeting St *SSR2* (Supplemental Figures 6A and 6B and Supplemental Tables 2 to 4), controlled by an estradiol-inducible promoter (Zuo et al., 2000). Transformants were treated with estradiol to induce TALEN expression. PCR and sequence analyses showed that TALEN could generate St *SSR2*

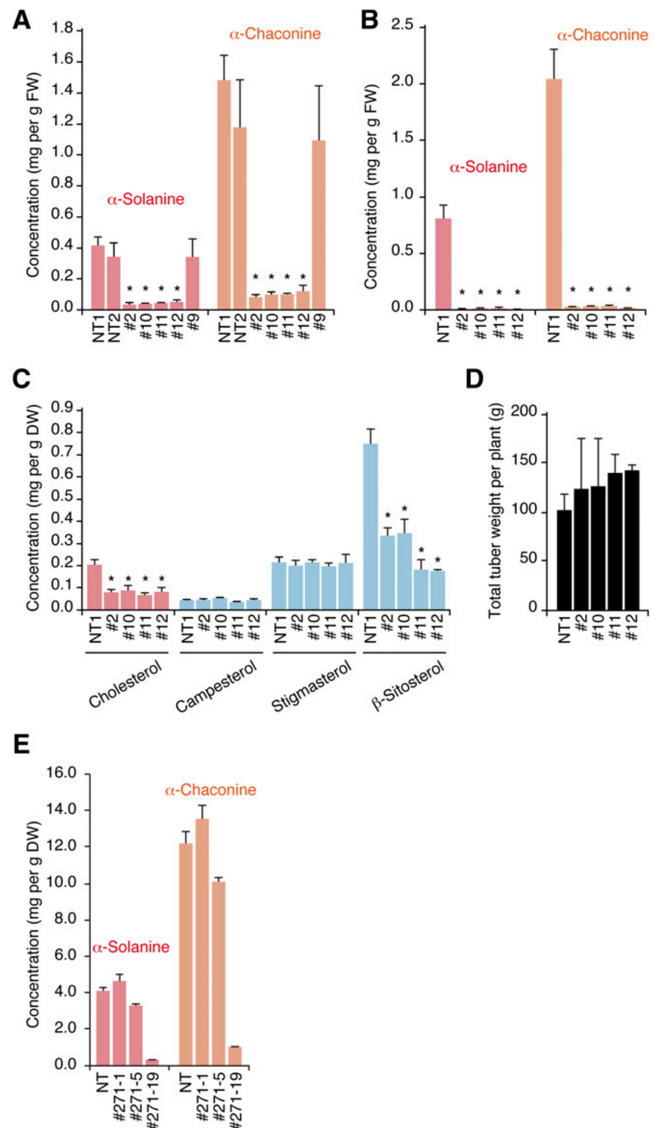

Figure 5. St *SSR2*-Silenced and -Disrupted Potato Plants.

(A) The predominant SGA (α-solanine and α-chaconine) levels in the stems of in vitro-grown nontransformed (NT) and St *SSR2*-silenced (#2, #10, #11, #12, and #9) plants (mean and SD, $n = 3$). Asterisks indicate values significantly different from the nontransformant (Dunnett's test, $P < 0.05$). FW, fresh weight.

(B) The predominant SGA levels in the peels of harvested tubers from nontransformed and St *SSR2*-silenced (#2, #10, #11, and #12) plants (mean and SD, $n = 3$). Asterisks indicate values significantly different from the nontransformant (Dunnett's test, $P < 0.05$). FW, fresh weight.

(C) Cholesterol and C-24 alkylsterol content in the stems of in vitro grown nontransformed and St *SSR2*-silenced (#2, #10, #11, and #12) plants (mean and SD, $n = 3$). Asterisks indicate values significantly different from the nontransformant (Dunnett's test, $P < 0.05$). DW, dry weight.

(D) Potato tuber yield of nontransformed and St *SSR2*-silenced (#2, #10, #11, and #12) plants (mean and SD, $n = 3$).

(E) The predominant SGA levels in the leaves of nontransformed and St *SSR2*-TALEN transformed (#271-1, #271-5, and #271-19) plants (mean and SD, $n = 3$). The predominant SGA levels of the nontransformant, #271-1, and #271-5 were significantly different from those of #271-19 (Dunnett's test, $P < 0.05$).

Downloaded from https://academic.oup.com/plcell/article/26/9/3763/6100345 by U.S. Department of Justice user on 16 August 2022

alleles with deletions or insertions of varied sizes at all four homologous chromosomes in the tetraploid genome of potato with no influence on St *SSR1* loci (Supplemental Figures 6C to 6E). The levels of predominant SGAs in the leaves of the St *SSR2*-disrupted plant (#271-19 in Figure 5E) were reduced to ~10% of those of the nontransformant (NT in Figure 5E) or transformants with some intact alleles of St *SSR2* (#271-1 and #271-5 in Figure 5E). The cholesterol level was also lower in the St *SSR2*-disrupted potato (#271-19 in Supplemental Figures 7A and 7B). Levels of some C-24 alkylsterol intermediates and C-24 alkylsterols such as 14α-methylcholesta-8,24-dien-3β-ol, 4α,14α-dimethylcholesta-8,24-dien-3β-ol, campesterol, and β-sitosterol in the St *SSR2*-disrupted line (#271-19 in Supplemental Figures 7A and 7B) were higher relative to those in the nontransformant (NT in Supplemental Figures 7A and 7B).

DISCUSSION

The data presented here show that St SSR2 is a key enzyme in the biosynthesis of toxic SGAs derived from cholesterol in potato. The methyl addition at C-24 of cycloartenol is the initial step in C-24 alkylsterol biosynthesis in plants. The presence of the alkyl group at C-24 that originates from this reaction is the structural characteristic differentiating C-24 alkylsterol from cholesterol. Therefore, cholesterol levels in plants are hypothesized to be modulated by SMT; this hypothesis is supported by the lower cholesterol level in transgenic plants that overexpress SMT and the higher cholesterol level in SMT knockout plants (Diener et al., 2000; Schaeffer et al., 2000; Sitbon and Jonsson 2001; Holmberg et al., 2002; Arnqvist et al., 2003). However, the considerable amounts of cholesterol and its derivatives, SGAs, in solanaceous plants have suggested that these plants have a mechanism to produce cholesterol that is distinct from other plants. The in vitro and in vivo analyses of both potato and tomato enzymes showed that solanaceous plants have two types of DWF1 homologs, SSR1 and SSR2, with different substrate preferences. SSR1 has $\Delta^{24(28)}$ reduction activity required for C-24 alkylsterol biosynthesis. On the other hand, SSR2 has $\Delta^{24(25)}$ reduction activity, which is essential for biosynthesis of cholesterol, an SGA precursor, as well as weak $\Delta^{24(28)}$ reduction activity. These observations suggest that solanaceous plants have obtained an additional DWF1 homolog and developed it into SSR2 to meet the high demand for cholesterol.

The much reduced levels of cholesterol and SGAs in two distinct loss-of-function potato plants, i.e., silencing by RNAi and disruption by TALEN, strongly support the identification of SSR2 as a key enzyme in their biosyntheses. The increases of some C-24 alkylsterols and their intermediates in the St *SSR2*-silenced and St *SSR2*-disrupted potatoes were probably due to switching of the biosynthetic flow from the cholesterol-SGA pathway to the C-24 alkylsterol pathway caused by lowered or abolished SSR2 activity. The different effects of St *SSR2* silencing and St *SSR2* disruption were observed in the levels of campesterol, β-sitosterol, and isofucosterol produced. These inconsistencies can be attributed to an off-target effect of St *SSR2*-RNAi on St *SSR1* transcription in the silenced lines (Supplemental Figure 4B). The TALEN-disrupted line #271-19 is considered to have only null or strong alleles of the St *SSR2* locus because there was no or little intact genomic DNA for this locus (Supplemental Figure 6C). The residual SGA accumulated by this line (Figure 5E) might result from the weak $\Delta^{24(25)}$ reduction activity of St SSR1 toward cycloartenol or desmosterol (Supplemental Figure 3), rather than leaky activity of St SSR2. Although roles for cholesterol and SGAs in plant growth and development have not been fully elucidated, the normal growth and development of St *SSR2*-RNAi and St *SSR2*-TALEN potatoes also suggests that cholesterol and SGAs may not be essential for the normal growth of potato (at least) under unstressed growth conditions.

In addition to SGAs in solanaceous plants, spirostan and/or furostan saponins such as dioscin in yam (*Dioscorea* spp; order Liliales) and trigoneosides in fenugreek (*Trigonella* spp; order Fabales) could also be biosynthesized via cholesterol as an intermediate (Bennett and Heftmann, 1965; Hardman and Fazli, 1972). Whether these plants have any genes homologous to *DHCR24* is unknown. The paralogous relationship between *SSR1* and *SSR2* in the *Solanum* genus suggests that cholesterol biosynthetic mechanisms in plants were developed by convergent evolution in each order, family, or genus. That is, the cholesterol biosynthetic pathway in other plants may differ from that in *Solanum*.

In this study, we identified SSR2 as an enzyme that is responsible for the initial reaction from C-24 alkylsterol biosynthesis to cholesterol biosynthesis on the pathway to the production of toxic SGAs in solanaceous plants. In addition, the lower level of SGAs in the St *SSR2*-disrupted potato by targeted genome editing with TALEN is evidence that the St *SSR2* gene identified in this study is an excellent target for breeding potatoes with decreased SGA levels. Breeding an St *SSR2*-knockout potato may also be possible by targeting induced local lesions in genomes (TILLING) (Muth et al., 2008); however, such methods that involve conventional mutagenesis would not rapidly generate new cultivars of important polyploid food crops, such as tetraploid potato. Our results from the St *SSR2*-TALEN transformants demonstrated that targeted genome editing with TALEN is able to generate potatoes with no remaining intact allele (Supplemental Figures 8A to 8C). An St *SSR2*-knockout potato without the transgene will be obtained by self-crossing the transformant (Supplemental Figure 8D). In the engineered potatoes, the absence of any increase in levels of intermediates between cholesterol and SGAs or their unexpected derivatives is expected because of the reduced supply of cholesterol as a common precursor of SGAs. In our analyses, there was no marked increase in the levels of intermediates (Supplemental Figure 7A). Further study of the *SSR2*-knockout potato will increase our understanding of the roles of cholesterol and SGAs in potato. Furthermore, as one of the possible gain-of-function applications of *SSR2*, overexpression of *SSR2* in plants producing cholesterol-derived saponins could increase the production of such saponins, i.e., dioscin, as a raw material for steroidal drugs.

METHODS

Chemicals

Authentic samples of desmosterol, α-solanine, α-chaconine, and α-tomatine were purchased from Sigma-Aldrich. Desmosterol was also purchased from

Downloaded from https://academic.oup.com/plcell/article/26/9/3763/6100345 by U.S. Department of Justice user on 16 August 2022

Avanti Polar Lipids. Cholesterol, campesterol, stigmasterol, and β-sitosterol were purchased from Tama Biochemical Co. Cycloartenol and cycloartanol were prepared by saponification of cycloartenyl ferurate (Wako Pure Chemical Industries) and the catalytic reduction of cycloartenol using H_2 and Pd/C, respectively. Isofucosterol, 24-methylenecholesterol, and 24-methylenecycloartanol were prepared by previously described methods (Fagerlund and Idler, 1960; Lee et al., 1992; Fujimoto et al., 1997).

Cloning of *SSR* cDNAs

The tomato cDNA template was prepared from mRNA isolated from a flowering-stage leaf of *Solanum lycopersicum* cv Micro-Tom using the SV Total RNA Isolation System (Promega) and SuperScript First-Strand Synthesis System for RT-PCR (Life Technologies). The potato cDNA template was prepared from mRNA isolated from sprouts of *Solanum tuberosum* cv Sassy using an RNeasy Plant Mini Kit (Qiagen). KOD Plus DNA polymerase (Toyobo), PrimeSTAR Max DNA Polymerase (Takara Bio), and PrimeSTAR HS DNA Polymerase (Takara Bio) were used for PCR. The full open reading frames of St *SSR1*, St *SSR2*, Sl *SSR1*, and Sl *SSR2* were PCR amplified with the primer sets 5′-CACCATGACAGATGTTCAGGCTCC-3′/5′-TCAATCTTCAGGCTCATCAACT-3′, 5′-CACCATGTCGGATGCTAAGGCCC-3′/5′-TCAATTCGCAGGTTCATCAG-3′, 5′-CTTGTGGTACCATGACAGATGTTCAGGCTCCCCCCCCTCG-3′/5′-CCATGAGACTCGAGTCAATCTTCAGGCTCATCAACTTCTG-3′, and 5′-AAGTTGGTACCATGTCGGATGCTAAGGCCCCCGTGGC-3′/5′-ATTCTTCTAGATCAATTTGCTTCAGGAGTCTCTTGTTCAG-3′, respectively. The PCR products of St *SSR1* and St *SSR2* were cloned into the Gateway entry vector pENTR/D-TOPO (Life Technologies) and transferred to the yeast expression vector pYES-DEST52 (Life Technologies) with a Gateway LR clonase reaction (Life Technologies). The PCR product of Sl *SSR1* was cloned into *Kpn*I and *Xho*I sites of the yeast expression vector pYES2 (Life Technologies). The PCR product of Sl *SSR2* was cloned into *Kpn*I and *Xba*I sites of pYES2.

Construction of a Desmosterol-Producing Yeast T21 and a 24-Methylenecholesterol-Producing Yeast T31

The yeast constitutive expression vector pTochigi 101 was constructed from pYO323 (Ohya et al., 1991) by inserting the *Saccharomyces cerevisiae* GPD promoter followed by a linker with *Not*I and *Asc*I recognition sites and the PGK1 terminator between the *Apa*I and *Sac*I sites of the vector (Yoshida et al., 2008). St *DWF5* cDNA containing a full open reading frame was PCR amplified with the primer set 5′-CACCAATGGCGGAGTCTCAGTTG-3′/5′-CTAATAAATTCCTGGTATGACCC-3′ and designed based on SGN-U269317 from a unigene database in the Sol Genomics Network (Bombarely et al., 2011). The PCR product was cloned into the pENTR/D-TOPO vector (Life Technologies) and subcloned between the *Not*I and *Asc*I sites of pTochigi101.

S. cerevisiae strain BY4742 *erg4 erg5* was constructed from BY4742 *erg4* (*MAT*α *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 erg4::KanMX4*; Open Biosystems) (Kelly et al., 2001) by replacing the *ERG5* ORF with a *ble^r* gene (Gueldener et al., 2002). The modified cassette was PCR amplified with primer sets 5′-TGTATTTGTTCCGCAATTTCC-3′/5′-CATTTTGTTAAAAGGTATTTATTGTCTATT-3′, 5′-ACATTTTGCTATTCCAAGACAATAAATACCTTTTAACAAAATGCAGCTGAAGCTTCGTACGC-3′/5′-TAAATATGATTTATTGTCTGGACAAAGTTCTGTTTTTCCCCATTAGCATAGGCCACTAGTGGATCTG-3′, and 5′-TAATGGGGAAAAACAGAACTTTG-3′/5′-TTGAACATAACGTCTTCATCTCC-3′.

Strains T21 and T31 were constructed by transforming BY4742 *erg4 erg5* and BY4742 *erg6* (*MAT*α *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 erg6::KanMX4*; Open Biosystems) (Kelly et al., 2001) with pTochigi101 containing the St *DWF5* cDNA.

Yeast in Vivo Assays

Yeast transformation was performed using a Frozen-EZ Yeast Transformation II Kit (Zymo Research) or a Gene Pulser Xcell electroporation system (Bio-Rad) according to the manufacturers' protocols. Yeast strains T21 and T31 described above were transformed with St *SSR1*, St *SSR2*, Sl *SSR1*, and Sl *SSR2* cDNAs cloned into pYES-DEST52 or pYES2. T21 and T31 transformed with intact pYES2 were used as empty vector controls. The transformants were cultured in yeast Synthetic Minimum Medium containing 2% glucose supplemented with -His/-Ura DO supplement (Takara Bio) for 1 d at 30°C. The cells were transferred to new medium of the same composition except for containing 2% (w/v) galactose instead of glucose to induce the recombinant SSRs and cultured for two more days. The harvested cells were lyophilized and extracted with ethyl acetate, and the extracts were analyzed by GC-MS after trimethylsilylation.

In Vitro Assay

S. cerevisiae BY4742 *erg4* (Open Biosystems) (Kelly et al., 2001) was transformed with St *SSR1*, St *SSR2*, Sl *SSR1*, and Sl *SSR2* cDNAs cloned into pYES-DEST52 or pYES2. BY4742 *erg4* transformed with intact pYES2 was used as an empty vector control. The transformants were cultured in yeast Synthetic Minimal Medium containing 2% glucose supplemented with -Ura DO supplement (Takara Bio) for 1 d at 30°C. The cells were transferred to new medium of the same composition except for containing 2% galactose instead of glucose to induce the recombinant SSRs and cultured for one more day. The in vitro enzyme activity assays using yeast homogenates were performed as previously reported (Waterham et al., 2001; Ciufo et al., 2011). Briefly, the harvested cells were suspended in 20 mM Tris-HCl, pH 7.5, containing 50 mM NaCl and disrupted with a Beadbeater (BioSpec Products) containing 0.5-mm glass beads. After removing the glass beads and cell debris by centrifugation at 3000*g* for 5 min, protein concentrations in the homogenates were measured with Quick Start Bradford Protein Assay Kit 2 (Bio-Rad). The assay mixture contained 100 mM Tris-HCl, pH 7.23, 1 mM DTT, 20 μM flavin adenine dinucleotide, 2 mM NADPH, 0.5 mg mL^{-1} BSA, 0.56 mg mL^{-1} homogenate protein, and 168 μM sterol substrate. The substrate was added from a 420 μM stock solution in 100 mM Tris-HCl, pH 7.23, containing 1.25% (w/v) methyl-β-cyclodextrin. After incubation for 24 h at 30°C, samples were saponified with KOH and ethanol for 1 h at 80°C and extracted with *n*-hexane. The extracts were washed with water and analyzed by GC-MS after trimethylsilylation.

St *SSR2*-Silenced Potatoes

A 269-bp fragment of St *SSR2* cDNA that did not match over 21 consecutive nucleotides with St *SSR1* was PCR amplified using the primer set 5′-GAGCTCTAGACCCTAGGAGGAAGATCCAG-3′/5′-GGATCCATATGCGTTTCTCATTCCAACAACA-3′. An RNAi binary vector pKT251 targeting the St *SSR2* gene was constructed from the binary vector pKT11 (Umemoto et al., 2001) by locating two St *SSR2* fragments in opposite orientations interposing the third intron of the *Arabidopsis thaliana At4g14210* gene under the control of the *cauliflower mosaic virus 35S* (*CaMV35S*) promoter in the T-DNA region (Supplemental Figure 4A). The RNAi binary vector pKT251 was transformed into *Agrobacterium tumefaciens* GV3110. Potatoes (*S. tuberosum* cv Sassy) were transformed using *Agrobacterium* GV3110 cells with pKT251 as previously reported (Monnma, 1990). In vitro-grown plants were cultured at 20°C under a 16-h-light/8-h-dark cycle. Thirty-nine transformants were individually selected by genomic PCR of the shoots with the primer set 5′-TAAAGCACGAGGAAGCGGT-3′/5′-GCACAACAGACAATCGGCT-3′ targeting the kanamycin resistance gene on the T-DNA region integrated into the potato genome. RT-PCR analyses of St *SSR1* and St *SSR2* were

Downloaded from https://academic.oup.com/plcell/article/26/9/3763/6100345 by U.S. Department of Justice user on 16 August 2022

performed using the primer sets 5′-TTAGGTTTTTCTTTGGATGGGC-3′/5′-TCACCCTGCCTCTTGTGCAG-3′ and 5′-CTCTGCTCAAAGCCACA-CAA-3′/5′-TCAATTCGCAGGTTCATCAG-3′ and total RNA prepared from stems of five independent lines of in vitro-cultured plants, #2, #10, #11, #12 and #9, and nontransformants. As a control, we used the potato *elongation factor 1*α gene (*EF1*α) and the primer pair 5′-ATTGGAAACG-GATATGCTCCA-3′/5′-TCCTTACCTGAACGCCTGTCA-3′ (Nicot et al., 2005). For the quantitative comparison of the transcripts, the cycles of PCR reactions were optimized to make sure of both clear visualization of the amplified products on an agarose gel and amplification in the exponential phase. The optimal cycles were 20 and 25 for St *SSR1*, 25 for St *SSR2*, and 20 for *EF1*α (Supplemental Figure 4B).

Sl *SSR2*-Silenced Tomatoes

A fragment of Sl *SSR2* cDNA that did not match over consecutive 21 nucleotides with Sl *SSR1* was PCR amplified using the primer set 5′-GAGCTCTAGATGTCGGATGCTAAGGCC-3′/5′-GGATCCATATGCA-TTCCTCTGGCCAAG-3′. An RNAi binary vector pKT262 targeting Sl *SSR2* was constructed with the *Sl SSR2* fragment in a way similar to the construction of pKT251. Tomatoes (*S. lycopersicum* cv Micro-Tom) were transformed using *Agrobacterium* GV3110 cells with pKT262 as previously reported (Sun et al., 2006). Integration of the T-DNA region into the tomato genomic DNA was investigated by genomic PCR of the tomato leaves targeting the kanamycin resistance gene on the T-DNA region integrated into the tomato genome.

St *SSR2*-Disrupted Potato

The St *SSR2*-disrupted potato was generated by targeted genome editing with TALEN (Cermak et al., 2011; Mahfouz et al., 2011). We constructed a new backbone vector, pGW-TAL-NC (Zeo), as follows. The region containing a TALEN scaffold was PCR amplified from pcDNA-TAL-NC (Sakuma et al., 2013) using the primer set 5′-CACCACTAGTAAA-AATGGCTTCCTCCCCTCCAAAGAAAA-3′/5′-TTAAAAGTTTATCTCACC-GTTATTA-3′ and cloned into the pENTR/D-TOPO vector (Life Technologies). The fragment was transferred to the pDEST17 vector (Life Technologies) with a Gateway LR reaction and subsequently cloned into pDONR/Zeo (Life Technologies) with a Gateway BP reaction to create pGW-TAL-NC (Zeo).

Three TALEN target sequences (SSR2_A, SSR2_B, and SSR2_C) were designed using the TAL effector-Nucleotide Targeter 2.0 website (Doyle et al., 2012; https://tale-nt.cac.cornell.edu) (Supplemental Table 2). TAL effector repeats were constructed by the six-module Golden Gate assembly method (Sakuma et al., 2013). For the single-strand annealing (SSA) assay, left and right regions of each target sequence were incorporated into pcDNA-TAL-NC to create pcDNA-SSA-SSR2_A_L, pcDNA-SSA-SSR2_A_R, pcDNA-SSA-SSR2_B_L, pcDNA-SSA-SSR2_B_R, pcDNA-SSA-SSR2_C_L, and pcDNA-SSA-SSR2_C_R. Among the three TALEN pairs whose activity was evaluated by SSA assays as described below (Supplemental Table 3), the most active TALEN pair, TAL-SSR2_C, was selected for potato transformation. TALEN assembly of SSR2_C was incorporated between the Esp3I sites of pGW-TAL-NC (Zeo) to obtain pGW-TAL-NC-SSR2_C_L and pGW-TAL-NC-SSR2_C_R.

To construct pKT271, an estradiol-inducible TALEN vector, we referred to pER8 (Zuo et al., 2000) and used a custom gene synthesis service to obtain two DNA fragments, N1-2 (*Asi*SI site-*G10-90* promoter-*XVE*-KpnI site) and N3 (*Kpn*I site-pea *rbcS E9* terminator-SmaI site-*LexA* operator –46 to +12 of the *CaMV35S* promoter-NotI and *Asc*I sites-*Arabidopsis Heat Shock Protein* [*At5g59720*] terminator-PmeI and *Eco*RI sites). N1-2 digested with *Asi*SI/*Kpn*I and N3 digested with *Kpn*I/*Eco*RI were cloned into *Hind*II/*Eco*RI-digested pKT11 (Umemoto et al., 2001) using an *Asi*SI-*Hind*III synthetic linker to produce pESTRA. N3 was digested with *Kpn*I/*Eco*RI and cloned into *Kpn*I/*Eco*RI-digested pBluescript SK vector without the *Pst*I-*Sac*I region to produce pN3. The TALEN left region was excised from pGW-TAL-NC-SSR2_C_L as a *Not*I/*Asc*I fragment and cloned into *Not*I/*Asc*I-digested pESTRA to obtain pESTRA#23. The TALEN right region was excised from pGW-TAL-NC-SSR2_C_R as a *Not*I/*Asc*I fragment and cloned into *Not*I/*Asc*I-digested pN3 to obtain pN3#24. The fragment containing the *LexA* operator, −46 to +12 of *CaMV35S* promoter-TALEN right region, was excised from pN3#24 as a *Sma*I/*Eco*RI fragment and cloned into *Pme*I/*Eco*RI-digested (*Sma*I and *Pme*I cleavages produce blunt ends) pESTRA#23 to construct the TALEN vector pKT271 (Supplemental Figure 6A).

The TALEN vector pKT271 was transformed into *Agrobacterium* GV3110. Potatoes (*S. tuberosum* cv Sassy) were transformed using *Agrobacterium* GV3110 cells with pKT271 as previously reported (Monnma, 1990). Twenty-nine transformants were individually selected by genomic PCR of the shoots with the primer set 5′-TAAAGCACGAGGAAGCGGT-3′/5′-GCACAACAGACAATCGGCT-3′ targeting the kanamycin resistance gene on the T-DNA region integrated into the potato genome. The selected transformants were cultured in plant boxes (Murashige and Skoog medium [Murashige and Skoog, 1962] containing 3% sucrose, 100 mg L^{-1} carbenicillin, and 0.8% agar) at 20°C under a 16-h-light/8-h-dark cycle.

To induce TALEN expression, 6- to 8-cm-tall subcultured shoots grown in plant boxes were dipped in a solution containing 10 μM 17β-estradiol and 0.02% Silwet L-77 and then transferred to soil. They were grown at 23°C under a 16-h-light/8-h-dark cycle.

Two weeks after the transgenic potatoes were transferred to soil, genomic DNA was extracted from TALEN-induced transgenic potato plants to detect somatic mutants in the target site. Regions surrounding the St *SSR2* target sites were amplified by PCR using the primer set 5′-TGTTCTCTGACACTGTTGTAGCACT-3′/5′-TCGAAGCATACATACCG-GTCATCAT-3′ and PrimeSTAR Max DNA polymerase (Takara Bio). Amplified products were separated on 5% polyacrylamide gels (Ota et al., 2013). Two samples (lines #271-5 and #271-19) had extra bands probably due to the formation of heteroduplexes within the target sites (Supplemental Figure 6C). The PCR products were cloned into the pCR4-Blunt-TOPO (Life Technologies) vector, and 8 and 15 clones were randomly selected for sequencing from lines #271-5 and #271-19, respectively. The presence of targeted deletions or insertions was confirmed in two of eight sequenced clones from line #271-5 and in all 15 sequenced clones from line #271-19 (Supplemental Figures 6D and 6E). The corresponding region of the St *SSR1* gene was also PCR amplified from line #271-19 (with the primer set 5′-TGTTCTCAGACACTG-TTGTGTCATA-3′/5′-TTGAAGCATATCTACCAGTCATGCA-3′), as the most homologous potential off-target site (Supplemental Figure 6B); however, no mutations were detected in any of the eight sequenced clones.

The levels of sterols and SGAs in TALEN-induced transgenic potato lines were analyzed 6 to 7 weeks after the transgenic potatoes were transferred to soil as described below.

Construction of SSA Reporter Plasmids and the SSA Assay Using HEK293T Cells

Construction of SSA reporter plasmids and the SSA assay were performed as previously described (Sakuma et al., 2013). SSA reporter plasmids for SSR2_A, SSR2_B, and SSR2_C TALENs and pGL4-SSA-SSR2_A, pGL4-SSA-SSR2_B, and pGL4-SSA-SSR2_C were constructed as follows: sense and antisense oligonucleotides (Supplemental Table 4) were annealed and inserted between the *Bsa*I sites of the pGL4-SSA vector. The SSA reporter plasmid for the negative control samples, pGL4-SSA-ZFA36, was constructed as previously described (Ochiai et al., 2010). pGL4-SSA-HPRT1_B-15 (Sakuma et al., 2013) was used as the SSA reporter plasmid for the positive control TALEN.

For the TALEN-positive control, TALEN expression vectors, pcDNA-SSA-HPRT1_B_L and pcDNA-SSA-HPRT1_B_R, were constructed as

Downloaded from https://academic.oup.com/plcell/article/26/9/3763/6100345 by U.S. Department of Justice user on 16 August 2022

previously described (Sakuma et al., 2013) and cotransfected with reporter plasmids as described herein.

GC-MS Analysis

GC-MS analyses were conducted with the same conditions as described before (Choi et al., 2014). MS spectra of trimethylsilylated authentic standards are shown in Supplemental Figure 2. 14α-Methylcholesta-8,24-dien-3β-ol, 4α,14α-dimethylcholesta-8,24-dien-3β-ol, 24-ethylcholesta-5,23-dien-3β-ol, and 24-ethyldesmosterol are tentatively identified compounds that were deduced by comparison of their mass spectra (Supplemental Figure 9) with published spectra and the relative retention times (Kornfeldt and Croon, 1981; Berman et al., 1986; Akihisa et al., 1988; Narumi et al., 2001; Wretensjö and Karlberg, 2002). The endogenous levels of sterols in potato and tomato plants were calculated by comparing the peak area values of molecular ions (cholesterol, 14α-methylcholesta-8,24-dien-3β-ol, 24-methylenecholesterol, 4α,14α-dimethylcholesta-8,24-dien-3β-ol, campesterol, stigmasterol, and β-sitosterol), $[M–90]^+$ formed by elimination of a trimethylsilanol (24-ethylcholesta-5,23-dien-3β-ol, 24-ethyldesmosterol, cycloartenol, and 24-methylenecycloartenol), or $[M–98]^+$ formed by McLafferty rearrangement of a side chain (isofucosterol) for the endogenous sterols with that of the molecular ion or $[M–90]^+$ for the internal standard, respectively.

Liquid Chromatography-Mass Spectrometry Analysis of α-Chaconine and α-Solanine in St *SSR2*-Silenced Potatoes

Fresh plant materials (100 mg) were homogenized with a mixer mill at 4°C in a 1 mL solution containing 80% (v/v) methanol and 0.1% (v/v) formic acid. For analyses of the levels of α-chaconine and α-solanine in the stems of in vitro-grown St *SSR2*-silenced plants, 10 μg brassinolide was added as an internal standard. After centrifugation, 25 μL of supernatant was diluted with 475 μL 0.1% (v/v) formic acid solution and filtered with a MultiScreen Solvinert (Millipore). An aliquot (10 μL) was analyzed by liquid chromatography-mass spectrometry (LC-MS) using 10 mM ammonium formate in water (pH 10):acetonitrile (2:3, v/v) as eluent at a flow rate of 0.2 mL min^{-1} at 40°C. LC-MS was performed with a Shimadzu LCMS-2010EV apparatus operating in ESI mode attached to an XBridge Shield RP18-5 column (150 mm × 2.1 mm i.d.; Waters). Quantifications of α-solanine and α-chaconine were calculated from the ratio of peak area at *m/z* 868 and 852 from positive ion scans using a calibration curve of authentic samples (with both coefficients of determination: $r^2 > 0.999$), respectively.

LC-MS Analysis of α-Chaconine and α-Solanine in St *SSR2*-Disrupted Potatoes

Extractions and LC-MS analyses of the plant materials were performed with the same method as previously described (Ohyama et al., 2013). Quantifications of α-solanine and α-chaconine were calculated from the ratio of peak area at *m/z* 868 and 852, respectively, from positive ion scans using a calibration curve of authentic α-solanine (with a coefficient-of determination: $r^2 > 0.992$).

LC-MS Analysis of α-Tomatine

The sample preparation procedures and LC-MS conditions were identical to those for LC-MS analysis of α-chaconine and α-solanine in St *SSR2*-disrupted potatoes described above. Quantification of α-tomatine was calculated from the ratio of peak areas at *m/z* 1034 from positive ion scans using a calibration curve of authentic α-tomatine (with a coefficient of determination: $r^2 > 0.998$).

Quantification of Brassinosteroids

Frozen potato leaves (~1 g fresh weight) were lyophilized and crushed to a fine powder with 10-mm ceramic beads by vortex and then soaked in 10 volume of extraction solvent (80% acetonitrile, 1% acetic acid) with stable isotope-labeled castasterone and brassinolide. After centrifugation, the supernatant was concentrated and reconstituted with 1% acetic acid. To remove interfering compounds, the extract was passed through an Oasis HLB column (Waters), Oasis MCX column, and Oasis WAX column equilibrated with 1% acetic acid, and brassinosteroids-containing fraction was eluted with 80% acetonitrile and 1% acetic acid, 80% acetonitrile and 1% acetic acid, and 80% acetonitrile, respectively. In each column step, the eluate was evaporated and reconstituted with 50% methanol. The castasterone and brassinolide were measured with UHPLC-Q-Exactive (Thermo Fisher Scientific) with an ODS column (AQUITY UPLC BEH C_{18}, 1.7 μm, 2.1 × 100 mm; Waters). Brassinosteroids were separated at a flow rate of 0.25 mL min^{-1} with linear gradients of solvent A (0.05% formic acid) and solvent B (0.05% formic acid in acetonitrile) set according to the following profile: 0 min, 90% A + 10% B; 20 min, 5% A + 95% B. Data were processed by Xcalibur software (version 2.2; Thermo Fisher Scientific).

Statistical Analysis

Dunnett's tests (Dunnett, 1955) were performed using the R Project for Statistical Computing software (R Core Team, 2013) with the multcomp package (Hothorn et al., 2008).

Phylogenetic Analysis

Sequence alignments (Supplemental Data Set 1) were constructed in MEGA5 (Tamura et al., 2011) using ClustalW (Thompson et al., 1994). The maximum likelihood tree was inferred in MEGA5 (Tamura et al., 2011) using Kimura's two-parameter model (Kimura, 1980) with a discrete gamma distribution (five categories) and the partial deletion option applied for gaps/missing-data treatment. Bootstrap values were performed with 1000 replications (Felsenstein, 1985). GenBank/EMBL/DDBJ accession numbers for the nucleotide sequences of the DHCR24 homologs are shown in Figure 2.

Multiple Alignments of Deduced SSR Amino Acid Sequences

Multiple alignments of deduced SSR amino acid sequences were constructed in GENETYX-MAC version 16 (Genetyx) with ClustalW2 (Larkin et al., 2007).

Accession Numbers

The sequences reported in this article have been deposited in the GenBank/EMBL/DDBJ database under the following accession numbers: AB839749 (St *SSR1*), AB839750 (St *SSR2*), AB839356 (Sl *SSR1*), AB839357 (Sl *SSR2*), and AB839751 (St *DWF5*). The accession numbers of the pER8 and potato *EF1α* in the GenBank/EMBL/DDBJ database are AF309825 and AB061263, respectively.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Modified Ergosterol Biosynthesis in Yeast Strains T21 and T31.

Supplemental Figure 2. MS Spectra of Authentic Sterol Standards.

Supplemental Figure 3. In Vitro Enzymatic Assays of SSRs.

Supplemental Figure 4. St *SSR2*-Silenced Potatoes.

Supplemental Figure 5. Sl *SSR2*-Silenced Tomatoes.

Supplemental Figure 6. TALEN Expression Vector Targeting St *SSR2*.

Downloaded from https://academic.oup.com/plcell/article/26/9/3763/6100345 by U.S. Department of Justice user on 16 August 2022

Supplemental Figure 7. St *SSR2*-Disrupted Potato.

Supplemental Figure 8. Flow Diagram of TALEN-Induced *SSR2* Knockout in Potato.

Supplemental Figure 9. MS Spectra of Sterols in Potato and Tomato.

Supplemental Table 1. Levels of Brassinosteroids in Leaves from Nontransformed and St *SSR2*-RNAi Transformed Potatoes.

Supplemental Table 2. TALEN Pairs Designed to Target St *SSR2*.

Supplemental Table 3. Functional Evaluation of Engineered TALENs Targeting the *SSR2* Gene by the SSA Assay.

Supplemental Table 4. Nucleotide Sequences of Oligonucleotides Used for Preparation of SSA Reporter Vectors.

Supplemental Data Set 1. Multiple Alignments Used to Construct the Phylogenetic Tree in Figure 2.

Supplemental Data Set 2. Multiple Alignments of Deduced SSR Amino Acid Sequences from Potato and Tomato.

Supplemental References.

ACKNOWLEDGMENTS

We thank Yoshinori Fujimoto (Tokyo Institute of Technology) and Shin-ichi Ayabe (Nihon University) for encouragement, Masaharu Mizutani (Kobe University) for helpful discussion, and Tomomi Sawada (RIKEN), Masako Otsuka, Ryo Negoya, Kaori Kuno, and Chie Yoshida (Kirin Co.) for technical support. Tomato cultivar Micro-Tom (TOMJPF00001) was provided by the University of Tsukuba, Gene Research Center, through the National Bio-Resource Project of the Education, Culture, Sports, Science, and Technology (MEXT), Japan. This study was supported in part by the Program for Promotion of Basic and Applied Researches for Innovations in Bio-oriented Industry (BRAIN), Japan; Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science; and the Scientific Technique Research Promotion Program for Agriculture, Forestry, Fisheries, and Food Industry, Japan.

AUTHOR CONTRIBUTIONS

S.S., K.O., S.Y., H. Seki, T.S., T.Y., M.K., H. Sakakibara, T.A., T.M., K.S., and N.U. designed experiments. S.S., K.O., S.Y., T.S., Y.T., M.K., and N.U. performed the experiments. S.S., K.O., S.Y., H. Seki, T.S., Y.T., M.K., H. Sakakibara, and N.U. analyzed the data. S.S., K.O., S.Y., H. Seki, T.S., M.K., H. Sakakibara, T.A., T.M., K.S., and N.U. wrote the article. All authors discussed the results and approved the article.

Received July 25, 2014; revised July 25, 2014; accepted August 19, 2014; published September 12, 2014.

REFERENCES

Akihisa, T., Ahmad, I., Singh, S., Tamura, T., and Matsumoto, T. (1988). 14α-Methylzymosterol and other sterols from *Wrightia tinctoria* seeds. Phytochemistry **27:** 3231–3234.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:** 3389–3402.

Arnqvist, L., Dutta, P.C., Jonsson, L., and Sitbon, F. (2003). Reduction of cholesterol and glycoalkaloid levels in transgenic potato plants by overexpression of a type 1 sterol methyltransferase cDNA. Plant Physiol. **131:** 1792–1799.

Bennett, R.D., and Heftmann, E. (1965). Biosynthesis of *Dioscorea* sapogenins from cholesterol. Phytochemistry **4:** 577–586.

Benveniste, P. (1986). Sterol biosynthesis. Annu. Rev. Plant Physiol. **37:** 275–308.

Berman, J.D., Goad, L.J., Beach, D.H., and Holz, G.G., Jr. (1986). Effects of ketoconazole on sterol biosynthesis by *Leishmania mexicana mexicana* amastigotes in murine macrophage tumor cells. Mol. Biochem. Parasitol. **20:** 85–92.

Bombarely, A., Menda, N., Tecle, I.Y., Buels, R.M., Strickler, S., Fischer-York, T., Pujar, A., Leto, J., Gosselin, J., and Mueller, L. A. (2011). The Sol Genomics Network (solgenomics.net): growing tomatoes using Perl. Nucleic Acids Res. **39:** D1149–D1155.

Bouvier-Navé, P., Husselstein, T., Desprez, T., and Benveniste, P. (1997). Identification of cDNAs encoding sterol methyl-transferases involved in the second methylation step of plant sterol biosynthesis. Eur. J. Biochem. **246:** 518–529.

Cermak, T., Doyle, E.L., Christian, M., Wang, L., Zhang, Y., Schmidt, C., Baller, J.A., Somia, N.V., Bogdanove, A.J., and Voytas, D.F. (2011). Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. Nucleic Acids Res. **39:** e82.

Choe, S., Dilkes, B.P., Gregory, B.D., Ross, A.S., Yuan, H., Noguchi, T., Fujioka, S., Takatsuto, S., Tanaka, A., Yoshida, S., Tax, F.E., and Feldmann, K.A. (1999). The Arabidopsis *dwarf1* mutant is defective in the conversion of 24-methylenecholesterol to campesterol in brassinosteroid biosynthesis. Plant Physiol. **119:** 897–907.

Choi, H., Ohyama, K., Kim, Y.Y., Jin, J.Y., Lee, S.B., Yamaoka, Y., Muranaka, T., Suh, M.C., Fujioka, S., and Lee, Y. (2014). The role of *Arabidopsis* ABCG9 and ABCG31 ATP binding cassette transporters in pollen fitness and the deposition of steryl glycosides on the pollen coat. Plant Cell **26:** 310–324.

Ciufo, L.F., Murray, P.A., Thompson, A., Rigden, D.J., and Rees, H.H. (2011). Characterisation of a desmosterol reductase involved in phytosterol dealkylation in the silkworm, *Bombyx mori.* PLoS ONE **6:** e21316.

Diener, A.C., Li, H., Zhou, W., Whoriskey, W.J., Nes, W.D., and Fink, G.R. (2000). *Sterol methyltransferase 1* controls the level of cholesterol in plants. Plant Cell **12:** 853–870.

Doyle, E.L., Booher, N.J., Standage, D.S., Voytas, D.F., Brendel, V.P., VanDyk, J.K., and Bogdanove, A.J. (2012). TAL Effector-Nucleotide Targeter (TALE-NT) 2.0: tools for TAL effector design and target prediction. Nucleic Acids Res. **40:** W117–W122.

Dunnett, C.W. (1955). A multiple comparison procedure for comparing several treatments with a control. J. Am. Stat. Assoc. **50:** 1096–1121.

Fagerlund, U.H.M., and Idler, D.R. (1960). Marine sterols. VII. Synthesis of 29-isofucosterol and the attempted synthesis of 17-dehydrocholesterol. J. Fish. Res. Board Can. **17:** 597–599.

Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. Evolution **39:** 783–791.

Friedman, M. (2002). Tomato glycoalkaloids: role in the plant and in the diet. J. Agric. Food Chem. **50:** 5751–5780.

Friedman, M. (2006). Potato glycoalkaloids and metabolites: roles in the plant and in the diet. J. Agric. Food Chem. **54:** 8655–8681.

Fujimoto, Y., Ohyama, K., Sato, N., Yamada, J., and Morisaki, M. (1997). 13C assignment of diastereotopic C-26 and -27 methyl groups of 24-methylenecholesterol: Steric course of hydrogen migration from C-24 to C-25 during its biosynthesis in higher plants. Chem. Pharm. Bull. (Tokyo) **45:** 224–226.

Grebenok, R.J., Galbraith, D.W., and Penna, D.D. (1997). Characterization of *Zea mays* endosperm C-24 sterol methyltransferase:

Downloaded from https://academic.oup.com/plcell/article/26/9/3763/6100345 by U.S. Department of Justice user on 16 August 2022

one of two types of sterol methyltransferase in higher plants. Plant Mol. Biol. **34:** 891–896.

Gueldener, U., Heinisch, J., Koehler, G.J., Voss, D., and Hegemann, J.H. (2002). A second set of *loxP* marker cassettes for Cre-mediated multiple gene knockouts in budding yeast. Nucleic Acids Res. **30:** e23.

Hardman, R., and Fazli, F.R.Y. (1972). Labelled steroidal sapogenins and hydrocarbons from *Trigonella foenumgraecum* by acetate, mevalonate and cholesterol feeds to seeds. Planta Med. **21:** 188–195.

Heftmann, E., Lieber, E.R., and Bennett, R.D. (1967). Biosynthesis of tomatidine from cholesterol in *Lycopersicon pimpinellifolium.* Phytochemistry **6:** 225–229.

Hirokawa, T., Boon-Chieng, S., and Mitaku, S. (1998). SOSUI: classification and secondary structure prediction system for membrane proteins. Bioinformatics **14:** 378–379.

Holmberg, N., Harker, M., Gibbard, C.L., Wallace, A.D., Clayton, J.C., Rawlins, S., Hellyer, A., and Safford, R. (2002). Sterol C-24 methyltransferase type 1 controls the flux of carbon into sterol biosynthesis in tobacco seed. Plant Physiol. **130:** 303–311.

Hothorn, T., Bretz, F., and Westfall, P. (2008). Simultaneous inference in general parametric models. Biom. J. **50:** 346–363.

Husselstein, T., Gachotte, D., Desprez, T., Bard, M., and Benveniste, P. (1996). Transformation of *Saccharomyces cerevisiae* with a cDNA encoding a sterol *C*-methyltransferase from *Arabidopsis thaliana* results in the synthesis of 24-ethyl sterols. FEBS Lett. **381:** 87–92.

Itkin, M., et al. (2013). Biosynthesis of antinutritional alkaloids in solanaceous crops is mediated by clustered genes. Science **341:** 175–179.

Itkin, M., et al. (2011). GLYCOALKALOID METABOLISM1 is required for steroidal alkaloid glycosylation and prevention of phytotoxicity in tomato. Plant Cell **23:** 4507–4525.

Jacobsen, E., and Rousselle, P. (1993). Potato. In Traditional Crop Breeding Practices: An Historical Review to Serve as a Baseline for Assessing the Role of Modern Biotechnology (Paris: Organisation for Economic Co-operation and Development), pp. 191–204.

Kauschmann, A., Jessop, A., Koncz, C., Szekeres, M., Willmitzer, L., and Altmann, T. (1996). Genetic evidence for an essential role of brassinosteroids in plant development. Plant J. **9:** 701–713.

Kelly, D.E., Lamb, D.C., and Kelly, S.L. (2001). Genome-wide generation of yeast gene deletion strains. Comp. Funct. Genomics **2:** 236–242.

Kim, T.W., Hwang, J.Y., Kim, Y.S., Joo, S.H., Chang, S.C., Lee, J.S., Takatsuto, S., and Kim, S.K. (2005). Arabidopsis CYP85A2, a cytochrome P450, mediates the Baeyer-Villiger oxidation of castasterone to brassinolide in brassinosteroid biosynthesis. Plant Cell **17:** 2397–2412.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. **16:** 111–120.

Klahre, U., Noguchi, T., Fujioka, S., Takatsuto, S., Yokota, T., Nomura, T., Yoshida, S., and Chua, N.H. (1998). The Arabidopsis *DIMINUTO/DWARF1* gene encodes a protein involved in steroid synthesis. Plant Cell **10:** 1677–1690.

Kornfeldt, A., and Croon, L.B. (1981). 4-Demethyl-, 4-monomethyl- and 4,4-dimethylsterols in some vegetable oils. Lipids **16:** 306–314.

Kozukue, N., and Mizuno, S. (1990). Effects of light exposure and storage temperature on greening and glycoalkaloids content in potato tubers. J. Jpn. Soc. Hortic. Sci. **59:** 673–677.

Larkin, M.A., et al. (2007). Clustal W and Clustal X version 2.0. Bioinformatics **23:** 2947–2948.

Lee, S.S., Young, L.H., and Wang, K.C. (1992). Separation of 24-methylenecycloartanol from cycloartenol via a chemical method. J. Nat. Prod. **55:** 644–648.

Mahfouz, M.M., Li, L., Shamimuzzaman, M., Wibowo, A., Fang, X., and Zhu, J.K. (2011). De novo-engineered transcription activator-like effector (TALE) hybrid nuclease with novel DNA binding specificity creates double-strand breaks. Proc. Natl. Acad. Sci. USA **108:** 2623–2628.

McCue, K.F., Allen, P.V., Shepherd, L.V., Blake, A., Maccree, M.M., Rockhold, D.R., Novy, R.G., Stewart, D., Davies, H.V. and Belknap, W.R. (2007). Potato glycosterol rhamnosyltransferase, the terminal step in triose side-chain biosynthesis. Phytochemistry **68:** 327–334.

McCue, K.F., Allen, P.V., Shepherd, L.V., Blake, A., Whitworth, J., Maccree, M.M., Rockhold, D.R., Stewart, D., Davies, H.V., and Belknap, W.R. (2006). The primary in vivo steroidal alkaloid glucosyltransferase from potato. Phytochemistry **67:** 1590–1597.

McCue, K.F., Shepherd, L.V.T., Allen, P.V., Maccree, M.M., Rockhold, D.R., Corsini, D.L., Davies, H.V., and Belknap, W.R. (2005). Metabolic compensation of steroidal glycoalkaloid biosynthesis in transgenic potato tubers: using reverse genetics to confirm the in vivo enzyme function of a steroidal alkaloid galactosyltransferase. Plant Sci. **168:** 267–273.

Moehs, C.P., Allen, P.V., Friedman, M., and Belknap, W.R., (1997). Cloning and expression of solanidine UDP-glucose glucosyltransferase from potato. Plant J. **11:** 227–236.

Monnma, T. (1990). Recent study for genetic engineering of soybean glycinin gene. Plant Tissue Cult. Lett. **7:** 57–63.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant. **15:** 473–497.

Muth, J., Hartje, S., Twyman, R.M., Hofferbert, H.R., Tacke, E., and Prüfer, D. (2008). Precision breeding for novel starch variants in potato. Plant Biotechnol. J. **6:** 576–584.

Narumi, Y., Noguchi, T., Fujioka, S., and Takatsuto, S. (2001). Identification of 4,4-dimethyl- and 4-monomethylsterols in seeds of *Setaria italic* and *Echinochloa frumentacea.* J. Oleo Sci. **50:** 53–56.

Nicot, N., Hausman, J.F., Hoffmann, L., and Evers, D. (2005). Housekeeping gene selection for real-time RT-PCR normalization in potato during biotic and abiotic stress. J. Exp. Bot. **56:** 2907–2914.

Nomura, T., Kushiro, T., Yokota, T., Kamiya, Y., Bishop, G.J., and Yamaguchi, S. (2005). The last reaction producing brassinolide is catalyzed by cytochrome P-450s, CYP85A3 in tomato and CYP85A2 in *Arabidopsis.* J. Biol. Chem. **280:** 17873–17879.

Ochiai, H., Fujita, K., Suzuki, K., Nishikawa, M., Shibata, T., Sakamoto, N., and Yamamoto, T. (2010). Targeted mutagenesis in the sea urchin embryo using zinc-finger nucleases. Genes Cells **15:** 875–885.

Ohya, Y., Goebl, M., Goodman, L.E., Petersen-Bjørn, S., Friesen, J.D., Tamanoi, F., and Anraku, Y. (1991). Yeast CAL1 is a structural and functional homologue to the DPR1 (RAM) gene involved in ras processing. J. Biol. Chem. **266:** 12356–12360.

Ohyama, K., Okawa, A., Moriuchi, Y., and Fujimoto, Y. (2013). Biosynthesis of steroidal alkaloids in Solanaceae plants: involvement of an aldehyde intermediate during C-26 amination. Phytochemistry **89:** 26–31.

Ota, S., Hisano, Y., Muraki, M., Hoshijima, K., Dahlem, T.J., Grunwald, D.J., Okada, Y., and Kawahara, A. (2013). Efficient identification of TALEN-mediated genome modifications using heteroduplex mobility assays. Genes Cells **18:** 450–458.

Quackenbush, J., Cho, J., Lee, D., Liang, F., Holt, I., Karamycheva, S., Parvizi, B., Pertea, G., Sultana, R., and White, J. (2001). The TIGR Gene Indices: analysis of gene transcript sequences in highly sampled eukaryotic species. Nucleic Acids Res. **29:** 159–164.

R Core Team (2013). R: A language and environment for statistical computing. (Vienna, Austria: R Foundation for Statistical Computing).

Downloaded from https://academic.oup.com/plcell/article/26/9/3763/6100345 by U.S. Department of Justice user on 16 August 2022

Sakuma, T., et al. (2013). Efficient TALEN construction and evaluation methods for human cell and animal applications. Genes Cells **18:** 315–326.

Schaeffer, A., Bouvier-Navé, P., Benveniste, P., and Schaller, H. (2000). Plant sterol-C24-methyl transferases: different profiles of tobacco transformed with *SMT1* or *SMT2.* Lipids **35:** 263–269.

Shi, J., Gonzales, R.A., and Bhattacharyya, M.K. (1996). Identification and characterization of an S-adenosyl-L-methionine: delta 24-sterol-C-methyltransferase cDNA from soybean. J. Biol. Chem. **271:** 9384–9389.

Sigrist, C.J.A., Cerutti, L., Hulo, N., Gattiker, A., Falquet, L., Pagni, M., Bairoch, A., and Bucher, P. (2002). PROSITE: a documented database using patterns and profiles as motif descriptors. Brief. Bioinform. **3:** 265–274.

Sitbon, F., and Jonsson, L. (2001). Sterol composition and growth of transgenic tobacco plants expressing type-1 and type-2 sterol methyltransferases. Planta **212:** 568–572.

Sun, H.J., Uchii, S., Watanabe, S., and Ezura, H. (2006). A highly efficient transformation protocol for Micro-Tom, a model cultivar for tomato functional genomics. Plant Cell Physiol. **47:** 426–431.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. **28:** 2731–2739.

Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:** 4673–4680.

Tomato Genome Consortium (2012). The tomato genome sequence provides insights into fleshy fruit evolution. Nature **485:** 635–641.

Tschesche, R., and Hulpke, H. (1966). Zur Biosynthese von Steroid-Derivaten im Pflanzenreich. 4. Mitt.: Biogenese von Tomatidin aus Cholesterin. Z. Naturforschg. **21b:** 893–894.

Tschesche, R., and Hulpke, H. (1967). Zur Biosynthese von Steroid-Derivaten im Pflanzenreich. 8. Mitt.: Biogenese von Solanidin aus Cholesterin. Z. Naturforschg. **22b:** 791.

Umemoto, N., Tsukahara, M., Yoshioka, M. (2001). JP Patent Publication (Kokai) 2001–161373 A.

Waterham, H.R., Koster, J., Romeijn, G.J., Hennekam, R.C., Vreken, P., Andersson, H.C., FitzPatrick, D.R., Kelley, R.I., and Wanders, R.J. (2001). Mutations in the 3β-hydroxysterol Δ^{24}-reductase gene cause desmosterolosis, an autosomal recessive disorder of cholesterol biosynthesis. Am. J. Hum. Genet. **69:** 685–694.

Wretensjö, I., and Karlberg, B. (2002). Characterization of sterols in refined borage oil by GC-MS. J. Am. Oil Chem. Soc. **79:** 1069–1074.

Xu, X., et al; Potato Genome Sequencing Consortium (2011). Genome sequence and analysis of the tuber crop potato. Nature **475:** 189–195.

Yoshida, S., Imoto, J., Minato, T., Oouchi, R., Sugihara, M., Imai, T., Ishiguro, T., Mizutani, S., Tomita, M., Soga, T., and Yoshimoto, H. (2008). Development of bottom-fermenting *Saccharomyces* strains that produce high SO_2 levels, using integrated metabolome and transcriptome analysis. Appl. Environ. Microbiol. **74:** 2787–2796.

Zuo, J., Niu, Q.W., and Chua, N.H. (2000). Technical advance: An estrogen receptor-based transactivator XVE mediates highly inducible gene expression in transgenic plants. Plant J. **24:** 265–273.

Downloaded from https://academic.oup.com/plcell/article/26/9/3763/6100345 by U.S. Department of Justice user on 16 August 2022