JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Ripening-Specific Stigmasterol Increase in Tomato Fruit Is Associated with Increased Sterol C-22 Desaturase (*CYP710A11*) Gene Expression

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Phytosterol content and composition and sterol C-22 desaturase (Le*SD1*; *CYP710A11*) transcript levels in pericarp tissue of 'Rutgers' tomato fruit were compared in the wild-type (wt) and isogenic lines of the nonripening mutants *nor* and *rin* at four stages of ripening/aging. Wild-type fruit were harvested at the mature-green (MG), breaker (BK), breaker plus 3 days (B + 3), and breaker plus 6 days (B + 6) stages, whereas *nor* and *rin* fruits were harvested at comparable chronological ages (days after pollination). At the MG stage, wt and mutant fruits had closely similar sterol contents, compositions, and conjugations, with >91% of the total sterols in the acylated steryl glycoside plus steryl glycoside (ASG + SG) fraction. During ripening/aging, there were substantial increases in total sterols and the percentage of sterols in the free plus esterified (FS + SE) fraction. Both changes were greater in wt than in *nor* or *rin*. In fruit of wt, *rin*, and *nor*, respectively, the increases in total sterols between MG and B + 6 were 2.1-, 1.9-, and 1.5-fold, and at B + 6 the percentages of total sterols in FS + SE were 42, 21, and 24. Among all sterol lipids (ASG, SG, FS, and SE), the ratio of stigmasterol (stigmasta-5,22-dien-3β-ol) to β-sitosterol (stigmast-5-en-3β-ol), the two major sterols in tomato, increased 2.3-fold during ripening of wt fruit but declined slightly during comparable aging of *nor* and *rin* fruits. In accord with these changes, the abundance of Le*SD1* transcript increased 4-fold in pericarp of ripening wt fruit, peaking at B + 3, whereas transcript levels in *nor* and *rin* fruits fluctuated but never exceeded the abundance in wt fruit at the MG stage. These findings indicate that the ripening-specific increase in stigmasterol in wt fruit results from a marked increase in Le*SD1* transcription and translation, which accelerates C-22 desaturation of the precursor sterol, β-sitosterol.

KEYWORDS: Tomato; *Solanum lycopersicum*; phytosterols; sterol 22-desaturase; gene expression; fruit ripening; nonripening mutants

INTRODUCTION

The 4-desmethyl C29 phytosterols β-sitosterol (stigmast-5-en-3β-ol) and stigmasterol (stigmasta-5,22*E*-dien-3β-ol), which differ by a double bond at C-22 of the alkyl side chain (**Figure 1**), are the predominant sterols in tomato (*Solanum lycopersicum*) and numerous other plants. Chow and Jen (*1*) reported a nearly 3-fold increase in the total sterol content of pericarp tissue during ripening of tomato fruit from mature green to red ripe, coincident with a dramatic increase in the ratio of stigmasterol to β-sitosterol. Duperon et al. (*2*) showed that tomato and related species in the genus *Solanum* are atypical in that leaves and other vegetative organs include a high proportion of their total phytosterols as the acylated steryl glycoside (ASG) and steryl glycoside (SG) conjugates (**Figure 1**), with much smaller amounts of free sterols (FS) and steryl fatty acyl esters (SE) (**Figure 1**). Whitaker (*3*) conducted a detailed analysis of changes in sterol content, composition, and conjugation during ripening of wild type (wt) and comparable aging of *nor* and *rin* nonripening mutant tomato fruits. As in leaves, ASG > SG were the predominant sterol lipids in pericarp tissue of mature-green tomato fruit, and it was found that all sterol lipid changes in ripening wt fruit were more pronounced than those in aging *nor* and *rin* fruits. In particular, the increase in the stigmasterol/sitosterol ratio, greatest in FS and least in SE, was largely ripening specific, as were substantial increases in the levels of SE > FS.

The occurrence of sterols including a trans double bond at C-22 of the alkyl side chain is limited to fungi, plants, and an array of microorganisms within the Protoctista (*4, 5*). In the yeast *Saccharomyces cerevisiae*, the *erg5* mutant was found to be deficient in sterol C-22 desaturase activity, producing ergosta-5,7-dien-3β-ol rather than ergosterol (ergosta-5,7,22*E*-trien-3β-

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10.1021/jf7037983 CCC: $40.75
Published on Web 05/08/2008

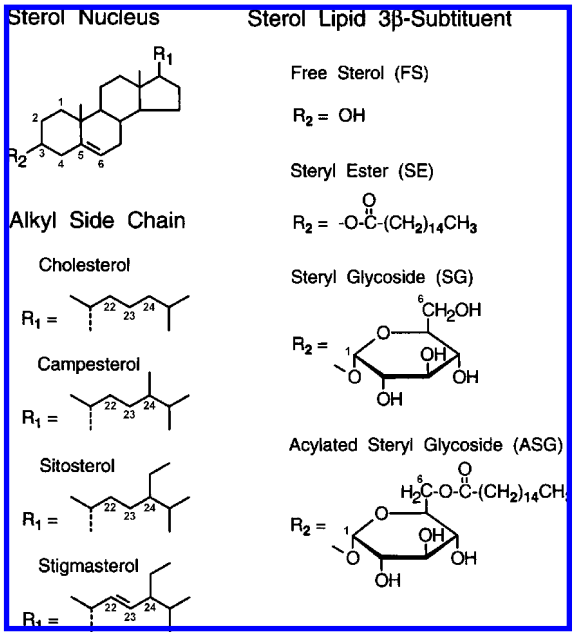

Figure 1. Structures of the four common desmethyl sterols (cholesterol, campesterol, sitosterol, and stigmasterol) and the four major classes of sterol lipids (FS, SE, SG, and ASG) in pericarp tissue of tomato fruit. All four desmethyl sterols share the same nucleus with one double bond at C-5(6). The alkyl side chains differ in the substituent at C-24 (−H, $-CH_3$, or $-CH_2CH_3$) and the presence or absence of a double bond at C-22(23). The 3β-OH in FS is esterified to a fatty acid in SE, glycosylated to (mainly) glucose in SG, and glycosylated to 6-*O*-fatty-acyl glucose in ASG.

ol) (*6*). This eventually enabled identification of the yeast sterol C-22 desaturase as a cytochrome P450 enzyme (CYP61) and cloning of *CYP61* encoded on chromosome XIII (*6–9*). Another decade passed before Morikawa et al. (*10*) were successful in cloning cytochrome P450 *CYP710A* gene family members encoding the sterol C-22 desaturase from higher plants. Specifically, four isogenes (*CYP710A1* through *CYP710A4*) were isolated from *Arabidopsis* and one gene from tomato (*CYP710A11*). Of these, *CYP710A1* and *CYP710A11* were heterologously expressed and the recombinant enzymes shown to convert β-sitosterol to stigmasterol. As well, gene overexpression and T-DNA knockout experiments in *Arabidopsis* demonstrated that *CYP710A2* is required for brassicasterol (ergosta-5,22*E*-dien-3β-ol) production and, along with *CYP710A1*, contributes to stigmasterol synthesis. Given the recent availability of the complete *CYP710A11* sterol C-22 desaturase cDNA from tomato (GenBank accession no. AB223043), the present study was conducted to determine if the dramatic, essentially ripening-specific, increase in stigmasterol content in tomato fruit pericarp tissue involves increased transcript abundance of *CYP710A11*.

MATERIALS AND METHODS

Plant Material and Tissue Sampling. Tomato plants (*S. lycopersicum* L.) of cv. Rutgers wt and isogenic lines of the nonripening mutants *nor* and *rin* were grown in a greenhouse under supplemental lighting using standard cultural practices as previously described (*11*). Flowers were tagged at anthesis and hand-pollinated. Only two fruits were allowed to develop from each inflorescence to ensure uniformity. Fruits from wt and mutant plants were compared at the same chronological age. The average days after pollination (DAP) at harvest were 45 ± 1, 48 ± 2, 51 ± 1, and 54 ± 1 for fruits at the mature-green (MG), breaker (BK), breaker plus 3 days (B + 3), and breaker plus 6 days (B + 6) stages, respectively. For wt, B + 3 and B + 6 fruits were at the pink and red stages of ripening. Fruits were cut longitudinally into six wedges after excision of the stem scar region and the locular tissue was removed. Outer pericarp sections (including the epidermis) from individual fruits were blotted, then quickly diced with a sharp knife, and frozen in liquid nitrogen. The tissue from each fruit was stored in a 50 mL plastic screw-cap tube at −80 °C until used for extraction of RNA and total lipids.

Lipid Extraction and Fractionation. Lipids in two pooled outer pericarp tissue samples from wt, *nor*, and *rin* fruits at each stage of ripening/aging (MG, BK, B + 3, B + 6) were extracted and analyzed. These pooled replicate samples, composed of approximately 1 g of frozen tissue from each of three fruits (total 3 g of fresh weight), were lyophilized for 48 h, and the dried tissue was pulverized into a fine powder. Each sample was then extracted twice with 15 mL of chloroform/methanol, 2:1. Two micrograms of cholestanol (5α-cholestan-3β-ol) in 20 μL of ethanol was added to the first extraction as an internal FS standard. For each extract, a phase separation was effected by the addition of 5 mL of aqueous 0.85% NaCl, followed by agitation and centrifugation for 2 min at 800*g*. The lower chloroform phases containing total lipids were combined, and the solvent was evaporated by a stream of N_2 with heating at 35 °C. The dried lipids were dissolved in 2 mL of chloroform prior to fractionation on a silicic acid column (100−200 mesh Bio-Sil A; Bio-Rad, Richmond, CA) as previously described (*3*). Briefly, neutral lipids (NL), including SE, FS, and most carotenoids, were eluted with 6 mL of chloroform plus 6.3 mL of chloroform/acetone, 20:1, and total glycolipids (GL), including ASG and SG, were eluted with 12.6 mL of acetone/methanol, 20:1.

Both the NL and GL column fractions were dried by N_2 evaporation and subjected to alkaline methanolysis by heating at 60 °C for 1 h with periodic vortex mixing in 1.5 mL of 600 mmol L^{-1} KOH in methanol. This procedure yielded FS from SE in the NL fraction and SG from ASG in the GL fraction, with conversion of most of the esterified fatty acids to fatty acyl methyl esters (FAME). After the addition of 1.5 mL of water, the NL fraction was neutralized with HCl and extracted twice with 3 mL aliquots of hexane. The hexane extracts were N_2 evaporated and dissolved in 1 mL of 2,2,4-trimethylpentane. The transesterified GL fraction was treated similarly, but extracted twice with 3 mL of chloroform to recover SG plus SG derived from ASG (ASG + SG fraction). FS and FS derived from SE in the NL fraction were separated from FAME and nonpolar carotenoids on a Pasteur pipet column containing silicic acid slurried in hexane. After loading of the sample in 2,2,4-trimethylpentane, FAME and nonpolar carotenoids were eluted with 4.2 mL of hexane/ethyl acetate, 20:1, followed by elution of FS and other NL of similar polarity with 5.5 mL of hexane/ethanol, 10:1 (FS + SE fraction).

Sterol and Steryl Glycoside Analysis. The generally low levels of sterols relative to pigments and other constituents in the FS + SE fraction necessitated digitonin precipitation and recovery, as described in ref *12*, prior to analysis by GC-FID. Sterol samples were dissolved in a small volume (20−40 μL) of 2,2,4-trimethylpentane, and 1 μL was injected into a Hewlett-Packard 5890 series II gas chromatograph equipped with a flame ionization detector and a 15 m HP-1 fused silica capillary column (0.25 mm i.d. and 0.25 μm film thickness). The column oven temperature was isothermal at 240 °C, and injector and detector temperatures were 280 and 300 °C, respectively. Helium was the carrier gas, with a column head pressure of 100 kPa. Data were collected and processed using ChemStation software (Agilent). Sterol standards, including cholesterol, cholestanol, campesterol, β-sitosterol, and stigmasterol, were purchased from Sigma-Aldrich and recrystallized from absolute ethanol prior to use. Their GC retention times were, respectively, 10.8, 11.0, 13.9, 15.1, and 17.3 min. Phytosterol concentrations in the samples were calculated from standard curves of the five commercial sterols using the ratio of the phytosterol peak area to the peak area of the internal standard, cholestanol (5α-cholestan-3β-ol). Unknown phytosterol peaks were arbitrarily treated as β-sitosterol.

SG in the de-esterified ASG + SG fraction were separated from other GL (e.g., glucocerebrosides) by thin-layer chromatography (TLC) on 20 × 20 cm glass plates precoated with a 250 μm thickness of silica gel 60 (EM Science, Darmstadt, Germany) and developed with the solvent mixture chloroform/methanol/acetic acid/water, 85:15:12.5:3.5, as previously described (*13, 14*). The TLC-purified SG samples were initially dissolved in 1 mL of methanol/chloroform, 16:1, and

quantified by C_{18}-HPLC-DAD with UV detection at 205 nm. HPLC analysis was performed with a Hewlett-Packard series 1100 HPLC system (Agilent Technologies) including a quaternary pump, autosampler, and diode array detector (DAD). Data were acquired using ChemStation software. Aliquots (80 μL) were injected onto a Luna 5 μm particle size C18(2) column (250 mm long, 4.6 mm i.d.) from Phenomenex (Torrance, CA) and eluted with isocratic methanol/acetonitrile, 60:40, at a flow rate of 1.0 mL/min over 25 min. A previously prepared tomato fruit SG sample of known concentration (determined spectrophotometrically as in refs *13* and *14*) was used as an external standard to quantify SG peaks. Coelution of campesteryl and stigmasteryl glucosides at 15.6 min, as noted by Breinhölder et al. (*15*), prevented use of HPLC analysis for determination of the stigmasterol/sitosterol ratio. Consequently, after the addition of 4 μg of cholestanol as an internal standard, FS were liberated from the remainder of the TLC-purified SG by acidic methanolysis prior to analysis by GC-FID as described for the FS + SE fraction. SG samples were dissolved in 1 mL of 2 mol L^{-1} HCl in methanol and heated at 75 °C for 90 min with periodic vortexing. After cooling, 1 mL of water was added, followed by neutralization with aqueous KOH and extraction twice with 3 mL of hexane to recover the liberated FS. The combined hexane phases were N_2 evaporated, the FS dissolved in 80 μL of 2,2,4-trimethylpentane, and 1 μL aliquots analyzed by GC-FID.

RT-PCR Amplification and Cloning of 'Rutgers' Le*SD1* cDNA. Total RNA was isolated as described by Gapper et al. (*16*) with modification. Frozen pericarp tissue samples (0.2 g) were ground to a powder in liquid N_2 using a mortar and pestle and transferred to 15 mL screw-cap centrifuge tubes, followed by the immediate addition of 0.25 mL of plant aid solution and 2 mL of lysis buffer from the RNAqueous RNA extraction kit (Ambion, Inc., Austin, TX). After vortexing and incubation for 10 min at 25 °C, the samples were processed according to the manufacturer's instructions. The resulting RNA preparations were treated with DNase (Promega) to degrade genomic DNA and extracted with phenol/chloroform, 1:1, and total RNA was precipitated by the addition of 1 volume of isopropyl alcohol plus 0.1 volume of 3 mol L^{-1} sodium acetate, pH 5.5. The precipitated RNA was pelleted by centrifugation, washed with cold 70% ethanol, and resuspended in nuclease-free water.

Total RNA isolated from pericarp tissue samples representing wt fruit at various stages of ripening was pooled, and cDNA was produced by reverse transcription (RT) using the Thermoscript RT-PCR system (Invitrogen) with a *CYP710A11* (AB223043) gene-specific primer (GSP), NEG173 (5′-AATGGGCAATCATCGTGTGCACC-3′), following the manufacturer's guidelines. The 1506 bp open reading frame (ORF) of Le*SD1* was amplified following two rounds of PCR. In the primary reaction, the outer GSPs NEG172 (5′-TTGTACGGTACTTCATCACATGGC-3′) and NEG173 were used with 1 μL of the pooled cDNA as a template. A second, nested reaction was then carried out using the inner GSPs NEG174 (5′-ATGGCATCCATTTGGGGTTTGTTATCTCC-3′) and NEG175 (5′-TCATCGTGTGCACCTGTGTGCAAGG-3′) with 1 μL of PCR product from the primary reaction as the template. Both PCR reactions were 30 cycles in length with a 55 °C annealing temperature and an extension for 2 min at 72 °C using the FastStart High Fidelity PCR System (Roche). The Le*SD1* amplicon from the second round of PCR was gel purified and cloned into the pGEM-T Easy vector (Promega) following the manufacturer's instructions.

Subsequent to cloning of the Le*SD1* ORF, cDNA produced by RT-PCR using the pooled tomato pericarp RNA and the Thermoscript RT-PCR system with the 3′ RACE adapter primer from the FirstChoice RLM-RACE kit (Ambion) was used to clone the 3′ untranslated region (3′-UTR) of Le*SD1*. Nested 3′ RACE was carried out to amplify a 0.4 kb cDNA fragment consisting of the terminal 203 bp of the Le*SD1* ORF plus the 3′-UTR. This was achieved by two rounds of PCR, the first employing the kit 3′ RACE outer primer plus the outer GSP 5′-GAGGAGAGACAAGAGGAGCG-3′ (Le*SD1* ORF nucleotides 1267−1286) and the second including the kit 3′ RACE inner primer plus the inner GSP 5′-TTCTAGCATTTGGTGCTGGG-3′ (Le*SD1* ORF nucleotides 1304−1313), following the manufacturer's instructions. Both rounds of PCR included 30 cycles, with annealing at 55 °C for 30 s, elongation at 68 °C for 60 s, and a final extension for 10 min at 72 °C. The 0.4 kb amplicon was then gel purified and cloned into the pGEM-T Easy vector (Promega) following the manufacturer's instructions.

Northern Analysis of Le*SD1* Expression. Total RNA was extracted from 6 g samples of frozen outer pericarp tissue from wt and nonripening mutant tomatoes (2 g from each of three fruits) in 10 mL of hot extraction buffer [100 mmol L^{-1} Tris-HCl, pH 8.0, 25 mmol L^{-1} EDTA, 2 mol L^{-1} NaCl, 2% CTAB (w/v), 2% PVP (w/v), 0.5 g/L spermidine), and 10 mL of preheated (80 °C) phenol (pH 8.0)]. Chloroform/isoamyl alcohol, 24:1 (10 mL), was then added, and the samples were mixed well prior to centrifugation at 10000*g* for 25 min. Total RNA was precipitated from the resulting supernatant by the addition of lithium chloride to a final concentration of 3 mol L^{-1} and subsequent storage at 4 °C overnight. The precipitated RNA was pelleted by centrifugation for 30 min at 10000*g*, and the pellet was then washed in 70% ethanol, air-dried, and resuspended in nuclease-free water. Northern analysis was carried out as described previously (*16*) using about 20 μg of total RNA per lane. After electrophoresis on a 1.2% agarose gel, RNA was transferred in 10× SSC to nylon membranes (Hybond XL, Amersham) by downward capillary transfer (*17*). After blotting, membranes were washed in 2× SSC and the RNA was fixed to membranes by UV cross-linking (GS Gene Linker UV Chamber, Bio-Rad). Double-stranded DNA probes for Le*SD1* or Le*18S* were prepared by random hexamer primed labeling using the 5′−3′ polymerase activity of the Klenow fragment of DNA polymerase (Roche) according to the manufacturer's instructions. The NEG174 and NEG175 primer pair was used to generate the full-length Le*SD1* ORF probe (1.5 kb), and the 0.3 kb Le*18S* probe was generated using the QuantumRNA Universal 18S Internal Standard primers from Ambion. Membranes were bathed in hybridization solution (*18*) and hybridized with [^{32}P]dATP-radiolabeled probes at 65 °C for 16 h. Membranes were washed sequentially for 20 min each in 2×, 1×, 0.5×, and 0.1× SSC plus 1% SDS (w/v) at 65 °C. After this series of washes, Kodak Biomax MS film was exposed to the membranes at −80 °C.

DNA Sequencing. Automated dideoxy sequencing was performed at the Iowa State University DNA Sequencing and Synthesis Facility. Reactions were set up using the Applied Biosytems (Foster City, CA) Prism BigDye Terminator v3.1 cycle sequencing kit with AmpliTaq DNA polymerase, and reactions were electrophoresed on an Applied Biosystems 3730 DNA analyzer. The programs BLASTN and BLASTP on the National Center for Biotechnology Information (NCBI) Website (www.ncbi/nlm/nih.gov) were used to compare the tomato *CYP710A11* and Le*SD1* cDNA and encoded protein sequences. The TBLASTN program was used to compare the Le*SD1* encoded protein sequence (GenBank accession no. ABX75534) with protein sequences derived by translation of tomato EST nucleotide sequences in the NCBI database.

RESULTS

Changes in Sterol Content and Conjugation. In the usual 9 day span from MG to B + 6, total phytosterols in the ASG + SG fraction in outer pericarp tissue of wt, *nor*, and *rin* fruits increased about 1.4-, 1.2-, and 1.7-fold, respectively (**Figure 2**, left). Most of the increase occurred between B + 3 and B + 6 in both *rin* and wt fruits, whereas it was more continuous and gradual in *nor* fruit. At the MG stage, the levels of FS + SE were similar and quite low (just over 3 μg/g of FW) in fruits of all three tomato lines, but rose sharply in the late stages of ripening/aging (**Figure 2**, right). FS + SE increased a total of 10.9-, 4.1-, and 4.7-fold in fruits of wt, *nor*, and *rin*, respectively, mostly after B + 3 in *nor* and *rin*, but between BK and B + 6 in wt. Sterol content and distribution among the four sterol lipid classes were closely similar in wt and mutant fruits at the MG stage, with >91% of the total sterols in the ASG + SG fraction. At B + 6 the percentages of total sterols in FS + SE were 42, 21, and 24 in fruits of wt, *nor*, and *rin*, respectively. The increase in total sterols (sum of ASG, SG, FS, and SE) over the full span of ripening/aging was 2.1-fold in wt (from 38.3 ± 1.4 to

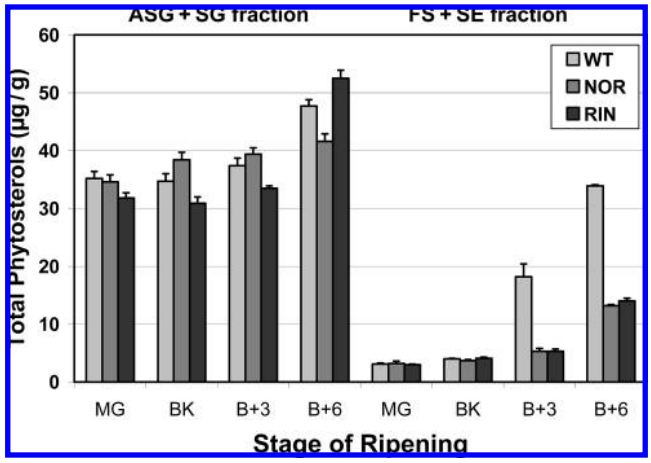

Figure 2. Total phytosterol concentration (μg/g of FW) in outer pericarp tissue of wt, *nor*, and *rin* fruits at four stages of ripening or comparable aging (MG, mature green; BK, breaker; B + 3, breaker plus 3 days; B + 6, breaker plus 6 days) in the acylated steryl glycoside plus steryl glycoside (ASG + SG) and free sterol plus steryl ester (FS + SE) fractions. Error bars indicate SD for analyses from two replicate tissue samples.

81.6 ± 0.9 μg/g of FW), 1.9-fold in *rin* (from 37.8 ± 2.6 to 54.8 ± 1.1 μg/g of FW), and 1.5-fold in *nor* (from 34.8 ± 0.8 to 66.5 ± 0.9 μg/g of FW).

Changes in Sterol Composition. Tables 1 and **2**, respectively, show the sterol composition of the FS + SE and ASG + SG fractions from outer pericarp tissue of wt, *nor*, and *rin* fruits at the four stages of ripening/aging. General trends in both combined sterol lipid fractions during ripening of wt fruit included a ≥2-fold decline in cholesterol accompanied by a ≥2-fold increase in campesterol. As well, the percentage of β-sitosterol declined substantially in both the FS + SE and ASG + SG fractions, whereas stigmasterol more than doubled in ASG + SG but decreased about 20% in FS + SE. Levels of cholesterol and campesterol generally changed less, and fluctuated more, with aging of *nor* and *rin* than with ripening of wt fruit. Overall, there was a slight decrease in cholesterol and a slight increase in campesterol in the ASG + SG fraction from both nonripening mutants (**Table 2**). The same trend was noted in FS + SE from *nor* fruit, whereas cholesterol increased substantially in FS + SE from *rin* fruit (**Table 1**). Percentages of both stigmasterol and β-sitosterol in the ASG + SG fraction increased modestly during aging of *nor* and *rin* fruits, and consistently levels of stigmasterol were a bit higher, and those of β-sitosterol a little lower, in *rin* compared with *nor* (**Table 2**). In the FS + SE fraction (**Table 1**), the patterns of change in stigmasterol and β-sitosterol levels differed with aging of *nor* and *rin* fruits, but, overall, there was a >2-fold decline in stigmasterol and a coincident marked increase in β-sitosterol. For FS + SE at the B + 3 and B + 6 stages, and for ASG + SG at the B + 3 stage, the abundance of sterols other than the four common desmethyl sterols was greater or much greater in wt than in *nor* or *rin* fruit (**Tables 1** and **2**).

Ratio of Stigmasterol to β-Sitosterol in FS + SE and ASG + SG. Somewhat surprisingly, the stigmasterol/sitosterol ratio at the MG stage was highest in *rin*, intermediate in wt, and lowest in *nor* fruits in both the FS + SE and ASG + SG combined sterol lipid fractions (**Figure 3**). However, during fruit ripening or comparable aging, the patterns of change in the ratio of these two major phytosterols were quite different in wt compared with the nonripening mutants. In the FS + SE fraction, there was a marked decline in the ratio in both *nor* and *rin* over the full span from MG to B + 6, although in *nor* fruit the change occurred after the BK stage, whereas in *rin* fruit there was an initial drop between MG and BK with a second sharp decline between B + 3 and B + 6 (**Figure 3A**). By contrast, the stigmasterol/sitosterol ratio in FS + SE from wt fruit decreased slightly between MG and BK but increased thereafter such that at B + 6 it was much higher in wt than in *nor* and *rin* (**Figure 3A**). In the ASG + SG fraction, which invariably included the majority of total phytosterols, the patterns of change in stigmasterol/sitosterol during ripening/aging differed somewhat from those in FS + SE (**Figure 3B**). There were slight fluctuations but overall very little change in the ratio with aging of nonripening mutant fruit from MG to B + 6. As in FS + SE, the ratio in ASG + SG was consistently higher in *rin* than in *nor*. In wt fruit, the stigmasterol/sitosterol ratio in ASG + SG rose substantially between BK and B + 3, with a further marked increase from B + 3 to B + 6.

Nucleotide and Encoded Amino Acid Sequences of Le*SD1*. Sequence analysis determined that the coding region of the 'Rutgers' tomato sterol C-22 desaturase cDNA (Le*SD1*; GenBank accession no. EU224275) is 1506 base pairs in length and encodes a predicted protein of 501 amino acids. Comparison of the coding region of Le*SD1* with the two other tomato sterol C-22 desaturase gene sequences in GenBank revealed 100% identity with the recently reported genomic sequence from

Table 1. Sterol Composition of the Free Sterol plus Steryl Ester Combined Fraction (FS + SE) in Outer Pericarp Tissue of wt, *nor*, and *rin* Tomato Fruits Harvested at Four Stages of Ripening or Comparable Aging (Days after Pollination)[a]

	mature green			breaker		
sterol	wt	*nor*	*rin*	wt	*nor*	*rin*
cholesterol	5.5 ± 0.2	5.7 ± 0.6	4.3 ± 0.0	4.1 ± 0.4	4.4 ± 0.3	5.1 ± 0.5
campesterol	6.0 ± 0.2	6.7 ± 0.2	5.7 ± 0.3	6.9 ± 0.2	5.1 ± 0.2	5.4 ± 0.2
stigmasterol	20.9 ± 0.9	16.5 ± 0.3	24.8 ± 0.6	17.8 ± 0.0	15.5 ± 0.3	16.6 ± 0.5
sitosterol	45.8 ± 0.6	51.7 ± 2.6	45.6 ± 2.0	47.3 ± 1.1	45.8 ± 2.1	45.8 ± 1.0
others	21.9 ± 1.4	19.6 ± 3.7	19.5 ± 1.7	24.0 ± 1.2	29.2 ± 2.3	27.1 ± 2.2

	breaker + 3 days			breaker + 6 days		
sterol	wt	*nor*	*rin*	wt	*nor*	*rin*
cholesterol	3.7 ± 0.2	4.8 ± 0.5	5.9 ± 0.4	2.8 ± 0.1	4.7 ± 0.2	7.5 ± 0.2
campesterol	8.8 ± 0.0	7.9 ± 0.9	8.3 ± 0.2	13.7 ± 0.4	7.0 ± 0.4	6.9 ± 0.1
stigmasterol	16.8 ± 0.9	14.3 ± 0.9	17.1 ± 0.9	16.7 ± 0.2	7.3 ± 0.3	10.9 ± 0.2
sitosterol	42.7 ± 1.7	54.1 ± 0.1	51.0 ± 0.5	31.8 ± 1.4	67.6 ± 1.0	64.9 ± 1.2
others	28.0 ± 1.0	19.0 ± 0.4	17.9 ± 0.2	35.0 ± 2.1	13.6 ± 1.0	10.0 ± 1.1

[a] Values are expressed as the mass percent of total sterols and represent the mean of analyses from two replicate tissue samples ± SD.

Table 2. Sterol Composition of the Acylated Steryl Glycoside plus Steryl Glycoside Combined Fraction (ASG + SG) in Outer Pericarp Tissue of wt, *nor*, and *rin* Tomato Fruits Harvested at Four Stages of Ripening or Comparable Aging (Days after Pollination)[a]

	mature green			breaker		
sterol	wt	*nor*	*rin*	wt	*nor*	*rin*
cholesterol	5.4 ± 0.1	3.2 ± 0.1	3.3 ± 0.1	3.6 ± 0.3	3.5 ± 0.3	5.5 ± 0.6
campesterol	7.0 ± 0.0	7.4 ± 0.0	7.2 ± 0.1	8.3 ± 0.1	6.9 ± 0.1	6.9 ± 0.5
stigmasterol	17.6 ± 0.2	16.4 ± 0.5	22.0 ± 0.3	17.7 ± 0.6	18.7 ± 0.2	21.5 ± 1.5
sitosterol	51.6 ± 1.3	54.9 ± 0.3	50.9 ± 0.3	55.1 ± 0.6	55.3 ± 0.5	54.4 ± 0.3
others	18.5 ± 1.5	18.2 ± 0.2	16.7 ± 0.2	15.2 ± 0.4	15.7 ± 0.5	11.8 ± 1.6

	breaker + 3 days			breaker + 6 days		
sterol	wt	*nor*	*rin*	wt	*nor*	*rin*
cholesterol	2.8 ± 0.1	3.2 ± 0.2	3.9 ± 0.3	2.0 ± 0.1	2.6 ± 0.1	2.9 ± 0.4
campesterol	10.2 ± 0.2	8.8 ± 0.2	9.9 ± 0.6	13.6 ± 0.4	8.2 ± 0.3	8.0 ± 0.2
stigmasterol	25.8 ± 1.8	19.3 ± 0.8	22.0 ± 1.0	36.1 ± 1.2	18.8 ± 0.4	24.4 ± 0.3
sitosterol	44.9 ± 1.1	58.1 ± 0.6	57.6 ± 0.7	35.3 ± 0.4	58.4 ± 1.2	54.6 ± 0.3
others	16.5 ± 0.8	10.7 ± 0.6	6.7 ± 1.5	12.1 ± 1.1	11.9 ± 2.1	10.3 ± 0.6

[a] Values are expressed as the mass percent of total sterols and represent the mean of analyses from two replicate tissue samples ± SD.

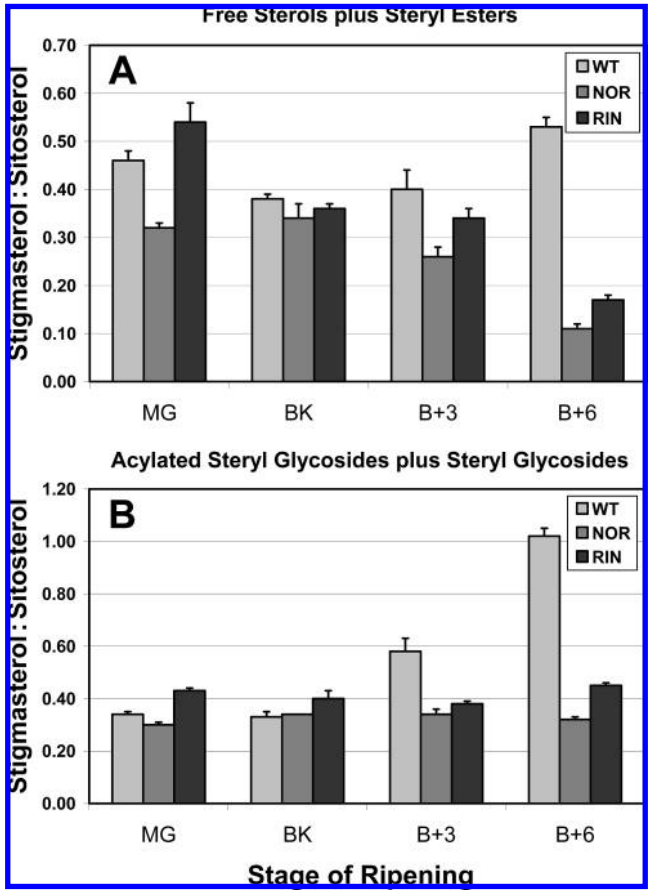

Figure 3. Ratio of stigmasterol to β-sitosterol in free sterols plus steryl esters (**A**) and acylated steryl glycosides plus steryl glycosides (**B**) in outer pericarp tissue of wt, *nor*, and *rin* fruits at four stages of ripening or comparable aging (MG, mature green; BK, breaker; B + 3, breaker plus 3 days; B + 6, breaker plus 6 days). Error bars indicate SD for analyses from two replicate tissue samples.

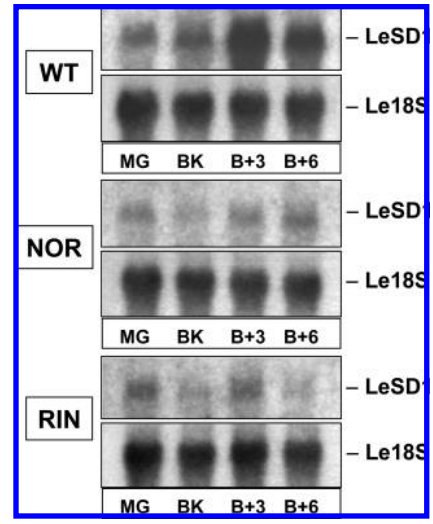

Figure 4. Northern analysis of Le*SD1* (*CYP710A11*) expression in outer pericarp tissue of wt, *nor*, and *rin* fruits at four stages of ripening or comparable aging (MG, mature green; BK, breaker; B + 3, breaker plus 3 days; B + 6, breaker plus 6 days). Blots of total pericarp tissue RNA separated on 1.2% agarose gels were hybridized with a 1.5 kb ^{32}P-labeled Le*SD1* cDNA fragment (entire coding region) and a 0.3 kb ^{32}P-labeled rRNA (*Le18S*) fragment. Quantitation of *LeSD1* transcript abundance is shown in **Figure 5**.

tomato chromosome 2 (AC215463; minus strand bp 38870-37365) and 99.7% identity (1501/1506 nucleotides) with the cDNA for *CYP710A11* (AB223043) cloned by Morikawa et al. (*10*). Comparison of the predicted amino acid sequence for Le*SD1* (ABX75534) with that of CYP710A11 (BAE93156) revealed 98.2% identity (479/501 amino acids), and the four nonconserved amino acid substitutions in Le*SD1* versus CYP710A11 were as follows: AA220, M/R; AA354, R/T; AA438, F/L; and AA458, F/I. None of these substitutions occur within the active site composed of AA303-315 with the sequence FLFAAQDASTSAL (*10*).

Le*SD1* Transcript Abundance and the Stigmasterol/Sitosterol Ratio. Northern analysis was used to follow expression of the 'Rutgers' sterol C-22 desaturase gene Le*SD1* in outer pericarp tissue of wt and nonripening mutant tomato fruits over the course of full ripening or comparable aging (**Figure 4**). The level of Le*SD1* transcript in wt fruit increased at least 4-fold during ripening from MG to B + 3 and then declined somewhat between B + 3 and B + 6 but still remained about 3-fold higher than the initial level at the MG stage (**Figures 4** and **5A**). In contrast, the abundance of Le*SD1* mRNA in *nor* and *rin* fruits changed only slightly with aging from MG to B + 6. There was some fluctuation in transcript levels in both nonripening mutants, but the patterns of change differed in *nor* and *rin*. Specifically, Le*SD1* expression in *nor* fruit decreased between

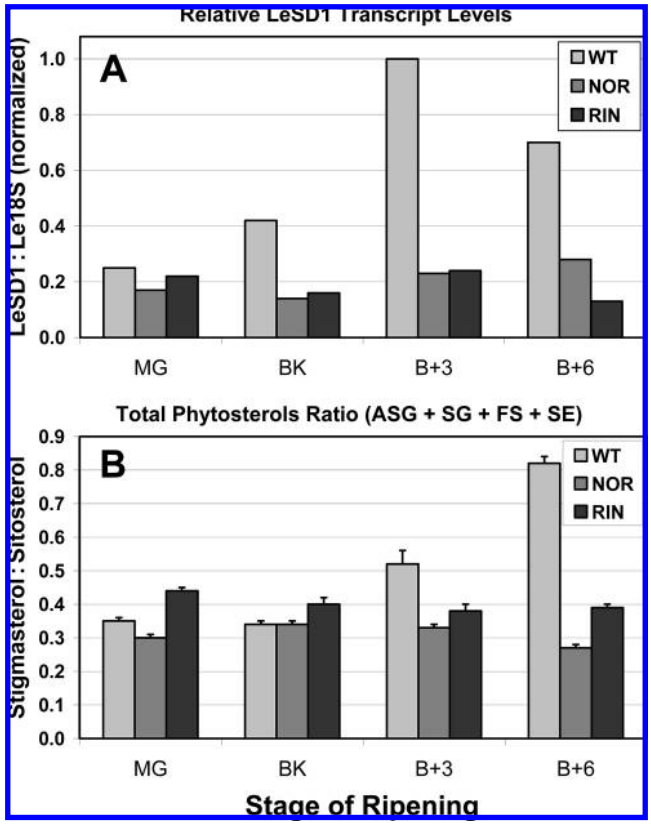

Figure 5. Relative abundance of *LeSD1* transcript (**A**) and ratio of stigmasterol to β-sitosterol in total phytosterols (**B**) in outer pericarp tissue of wt, *nor*, and *rin* fruits at four stages of ripening or comparable aging (MG, mature green; BK, breaker; B + 3, breaker plus 3 days; B + 6, breaker plus 6 days). Bars in **A** indicate the relative intensity of the Le*SD1* and Le*18S* rRNA loading control bands (expressed as Le*SD1*/Le*18S*) determined by densitometry scanning of the Northern blot images shown in **Figure 4**. Values were normalized, with the highest (wt at B + 3) set to 1.0. Total phytosterols in **B** included the four combined sterol lipids, acylated steryl glycosides (ASG), steryl glycosides (SG), free sterols (FS), and steryl esters (SE), and error bars indicate SD for analyses from two replicate tissue samples.

MG and BK and then increased about 2-fold with aging from BK to B+6, whereas in *rin* fruit there was a decrease between MG and BK, followed by a return to the initial level between BK and B + 3 and a subsequent drop to very low abundance by B + 6 (**Figures 4** and **5A**). Comparison of changes in Le*SD1* transcript levels and the stigmasterol/sitosterol ratio in total phytosterols (including ASG, SG, FS, and SE) over the course of fruit ripening/aging showed a good correlation from the standpoint that both parameters increased substantially in wt, whereas each exhibited only modest changes in *nor* and *rin* (**Figure 5**). In pericarp of wt fruit there was a clear lag of about 3 days between increased Le*SD1* expression and a corresponding increase in the ratio of stigmasterol to β-sitosterol. Comparison of fruits of *nor* and *rin* showed some discrepancy inasmuch as the stigmasterol/sitosterol ratio was consistently higher among sterol lipids in *rin*, whereas the Le*SD1* transcript was equally abundant in the two nonripening mutants at B + 3 and about 2-fold higher in *nor* at B + 6.

DISCUSSION

As noted in two prior studies (*1, 3*), substantial changes in sterol content, composition, and conjugation were associated with ripening of tomato fruit pericarp tissue. Specifically, there were increases in sterol accumulation, in the amounts of stigmasterol and campesterol at the expense of β-sitosterol, and in the percentage of total sterols in the FS + SE fraction. The increase in total sterol content observed in the present study was greater than that previously found for 'Rutgers' (*3*) but smaller than that reported for 'Walters' (*1*) wt fruit. Moreover, in this investigation sterol content in wt, *rin*, and *nor*, respectively, increased about 2.1-, 1.9-, and 1.5-fold from MG to B + 6, whereas previously (*3*) increases of only 1.6-fold in wt and 1.2-fold in *rin* were noted with ripening/aging. Many factors including cultivar, growing season, greenhouse versus field production, ripening on the plant versus postharvest, day/night temperature, and light intensity and spectral quality could contribute to these discrepancies.

With regard to light effects, Chow and Jen (*1*) found that postharvest ripening under red light rather than in the dark advanced the rise in sterol accumulation, induced an additional 10% increase in total sterols, and promoted a specific increase in campesterol. As well, ripening under far-red light reduced sterol accumulation, indicating that light effects on sterol biosynthesis are mediated by phytochrome. It is possible that changes in sterol C-24 methyl transferase (SMT) gene expression and enzyme activity contribute to both the general increase in sterol synthesis and the specific increase in campesterol. *SMT1* was shown to control the flux of carbon into sterol biosynthesis in tobacco seed (*19*), whereas cosuppression of *SMT2-1* in *Arabidopsis* markedly increased the campesterol/sitosterol ratio (*20*).

As mentioned above, the increase in total sterols with ripening of 'Rutgers' wt fruit was accompanied by a large increase in the ratio of FS + SE to ASG + SG. By comparison, increases in total sterols and in the percentage of FS + SE were smaller with comparable aging of *rin* and *nor* fruits. It is not understood why mature-green fruit as well as leaves and other tissues of *S. lycopersicum* include such a high percentage of total sterols as the ASG and SG conjugates. It has been proposed that these glycosides have some specialized function in cell membranes and in responses to various types of stress (*2, 21–23*). Clearly, 3β-hydroxysterol glucosyltransferase (SGT) and steryl glucoside 6′-*O*-acyltransferase (SGAT) activities must be high in developing tomato fruit and may decline with ripening and senescence (*2, 3*). This warrants further investigation, and the recent cloning of a small family of SGTs from the Solanaceous species *Withania somnifera* (*23*) should expedite discovery of SGT genes in tomato. There have been relatively few studies of ASG biosynthesis, and at present there appears to be no known SGAT gene sequence.

ASG, SG, and FS are membrane structural components, whereas SE are not (*22, 24–26*). SE appear to play a role in membrane homeostasis by excluding a pool of sterols, typically enriched in biosynthetic intermediates, from cell membranes (*24–26*). A number of studies have shown that levels of stigmasterol are relatively low in SE compared with other sterol lipid classes (*2, 3, 26*), and recently stigmasterol was found to be the poorest substrate among the common desmethyl sterols for a phospholipid:sterol acyltransferase (PSAT) overexpressed in *Arabidopsis* (*27*). The decline in the stigmasterol/sitosterol ratio in the FS + SE fraction during aging of *nor* and *rin* fruits is most likely a reflection of the increasing percentage of SE, and SE from wt 'Rutgers' fruit were previously shown to include the least stigmasterol among the four sterol lipid classes (*3*). An increase in SE has been noted with aging and senescence of various plant tissues and is thought to be associated with the increasing level of membrane disorganization (*2, 22, 26*). The

much higher level of SE in ripened wt compared with nonripening mutant fruit of comparable age (*3*) may result from both a higher rate of sterol synthesis and a more advanced stage of senescence.

With the recent cloning and characterization of sterol C-22 desaturase genes from *Arabidopsis* (*CYP710A1*, *CYP710A2*, *CYP710A3*, *CYP710A4*) and tomato (*CYP710A11*), all genes of the sterol biosynthetic pathway in higher plants have now been identified and isolated (*10, 24, 28*). Functional expression of *CYP710A1* and *CYP710A11* demonstrated that the products of these genes desaturate sitosterol to yield stigmasterol, and overexpression of *CYP710A1* and *CYP710A11* in *Arabidopsis* provided clear evidence of the same activity in vivo (*10, 28*). Le*SD1* (EU224275), the sterol C-22 desaturase cDNA cloned in the present study using RNA from 'Rutgers' tomato fruit pericarp and primers based on the 5′ and 3′ ends of the *CYP710A11* coding region, almost certainly represents the same gene as *CYP710A11* (AB223043), differing by only five nucleotides in the open reading frame and having a high degree of identity in the 3′-UTR as well. The presence of a small family of *CYP710A* genes in *Arabidopsis* that are differentially expressed in various tissues (*10*) suggests that the same may be true in tomato and other plants. However, a TBLASTN search of tomato ESTs on the NCBI Website using the LeSD1 deduced amino acid sequence (ABX75534) as bait identified sequences from fruit (DB711336), leaf (DB690920), flower bud (AW737712), and root (AW622568) tissues encoding ≥151 identical amino acids from the N terminus inward. This indicates that Le*SD1* is expressed in all of these tissues, and most likely is the principal or sole gene responsible for conversion of β-sitosterol to stigmasterol. Our results showing a correlation between sharply rising Le*SD1* transcript levels and a subsequent marked increase in the stigmasterol/sitosterol ratio during ripening of wt tomato fruit support this conclusion, at least for fruit pericarp tissue. Conversely, the minimal changes in Le*SD1* transcript abundance and the stigmasterol/sitosterol ratio during aging of *nor* and *rin* fruits are another indication that LeSD1 activity yields the large ripening-specific increase in stigmasterol in wt fruit.

Sterol C-22 desaturation is common to many organisms, but the function is currently unknown. The *erg5* (*CYP61*; *Erg5p*) mutation in yeast is not lethal (*6, 8, 9*), and the only observed phenotypes were low resistance to the polyene antibiotic nystatin (*6*) and increased sensitivity to osmotic stress (*8*). In *Arabidopsis*, neither overexpression of four individual CYP710A genes, which increased stigmasterol levels substantially, nor T-DNA knockout of CYP710A2, which abolished ergosta-5,22*E*-dien-3β-ol synthesis, visibly altered the phenotype. A number of studies have provided evidence that fluctuation in the stigmasterol/sitosterol ratio plays a role in responses to biotic and abiotic stress (*13, 28, 29*). Plant cell plasma membrane is greatly enriched in phytosterols, which are important in the modulation of bilayer fluidity and permeability, and also function in the formation of lipid microdomains ("rafts") and regulation of proton ATPase activity (*30, 31*). Stigmasterol and β-sitosterol were shown to have markedly different effects on the permeability, ordering, and fluidity of plant phospholipid vesicles (*32*), but nevertheless the two sterols have a similar ability to promote microdomain formation (*30, 33*). Considering the dramatic increase in the stigmasterol/sitosterol ratio with ripening of tomato fruit, future use of wt and nonripening mutant tomato lines as an experimental system may shed some light on the significance of sterol C-22 desaturation with respect to physiology and membrane function. Tomato fruit should also be useful in studies of other aspects of plant sterol lipid metabolism, particularly as the International Tomato Genome Sequencing Project progresses.

ACKNOWLEDGMENT

We thank Frances Trouth for her invaluable technical assistance in growing and processing the plant material and in Northern blot analysis of Le*SD1* transcript levels.

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Received for review December 31, 2007. Revised manuscript received February 20, 2008. Accepted March 18, 2008. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

JF7037983