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Involvement of Ethylene in the Accumulation of Esculeoside A during Fruit Ripening of Tomato (*Solanum lycopersicum*)

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The composition of glycoalkaloids in tomato fruit changes with ripening. However, it has not been clarified whether the accumulation of glycoalkaloids is controlled by the ripening-inducing phytohormone, ethylene. Here, we report the effect of ethylene on the accumulation of tomato fruit glycoalkaloids. We investigated the effect of exogenously applied ethylene. In response to ethylene treatment, the content of α-tomatine decreased, whereas the content of esculeoside A increased. Next, we analyzed the fruits of ripening mutants, *rin*, *nor*, and *Nr*. In fruits of these mutant lines, the level of accumulation of esculeoside A decreased, whereas α-tomatine accumulated to higher levels than in wild-type fruit. These results demonstrated that the esculeoside A accumulation was associated with production and perception of ethylene. Additionally, the accumulation profiles of the intermediate metabolites of esculeoside A biosynthesis in ripening mutant fruits suggest that a glycosylation step in the putative pathway from α-tomatine to esculeoside A depends on ethylene.

KEYWORDS: ***Solanum lycopersicum*; solanaceae; tomato; ethylene; ripening; glycoalkaloids; esculeoside A; α-tomatine**

INTRODUCTION

Solanaceous plants such as potato, tomato, and eggplant contain glycoalkaloids that are unique to the respective species (*1*−*4*). Among them, α-solanine and α-chaconine in potato and α-tomatine in tomato are reported to exhibit toxicity against a variety of fungi, insects, and animals (*2, 5*). In contrast, potentially beneficial health effects, such as growth inhibition of cancer cells (*6, 7*), inactivation of the *Herpes* virus (*8*), and lowering of cholesterol levels (*9*), have also been reported recently. Due to the dual nature of their biological activities, glycoalkaloid content has been one of the major interests for consumers concerned with the quality of potatoes, tomatoes, and their processed products (*2, 5, 10, 11*).

In tomato (*Solanum lycopersicum*), contents of α-tomatine (**1**) (**Figure 1**) and dehydrotomatine have been extensively analyzed, indicating that they depend on the cultivar, tissue, growth conditions, and growing region (*1, 12, 13*). The content of α-tomatine (**1**) is high in immature green fruit and drastically decreases in ripe fruit (*5, 14*−*17*). From ripe and overripe fruits, esculeoside A (**2**), esculeoside B, lycoperosides, and 3*β*-

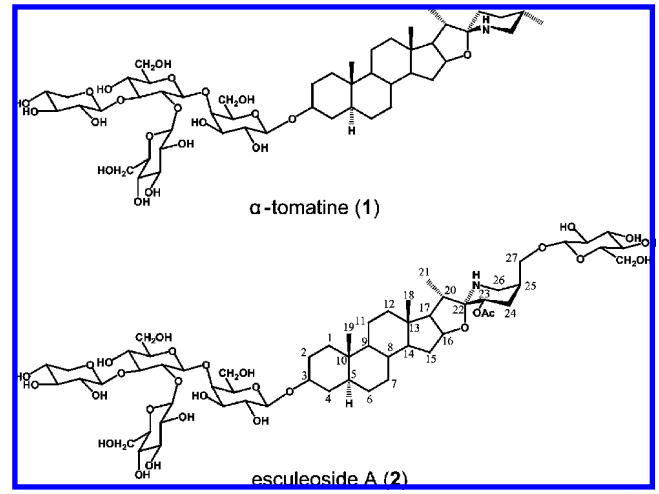

Figure 1. Structures of α-tomatine (**1**) and esculeoside A (**2**).

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hydroxy-5α-pregn-16-en-20-one lycotetraoside were isolated (*18*−*20*). It has been reported that the content of esculeoside A (**2**) (**Figure 1**), the main glycoalkaloid in ripe fruit (*21*), is affected by seasonal variation and ripening stages (*3*). A tentative metabolic pathway from α-tomatine (**1**) to esculeoside A (**2**) has been proposed recently, in which sequential hydroxylation, acetylation, hydroxylation, and glycosylation of α-tomatine (**1**) occur (*22*, *23*). In climacteric fruit, such as tomato, the phytohormone, ethylene, plays a central role in the induction of fruit ripening (*24*). During ripening, remarkable metabolic changes occur in tomato fruit (*25*). Along with the changes in central carbon metabolism and cell wall metabolism, secondary metabolism, such as carotenoid biosynthesis, is remarkably activated. This ripening-associated increase in the level of carotenoids is not observed in fruits of ripening mutants *ripening-inhibitor* (*rin*), *non-ripening* (*nor*), and *Never-ripe* (*Nr*) (*26*). The *rin* locus encodes a transcription factor, LeMADS-RIN, belonging to the MADS box family (*27*), and the *nor* locus encodes a transcription factor, NOR; both proteins are involved in developmental control of ethylene production (*28*, *29*). The *Nr* locus encodes a homologue of the *Arabidopsis* ethylene receptor with impaired ethylene binding capacity (*30*); thus, the *Nr* mutant fails to ripen due to insensitivity to ethylene. The lack of a ripening-associated increase in the level of carotenoids in these mutants indicates that carotenoid accumulation depends on ethylene. Ethylene signaling-dependent changes in the expression of carotenoid biosynthesis genes have been elucidated recently (*31*). However, whether glycoalkaloid accumulation depends on ethylene has not been clarified.

In this study, we investigated profiles of glycoalkaloids in fruits at different ripening stages, as well as in fruits treated with exogenously applied ethylene, using ultraperformance liquid chromatography time-of-flight mass spectrometry (UPLC-TOF-MS). The involvement of ethylene in the accumulation of esculeoside A (**2**) was further tested by using fruits of *rin*, *nor*, and *Nr*. We then analyzed putative structures and developmental profiles of glycoalkaloids that are hypothesized to be intermediates in the biosynthetic pathway between α-tomatine (**1**) and esculeoside A (**2**).

MATERIALS AND METHODS

Plant Materials. Tomato (*S. lycopersicum*) cv. Carol 10 was grown hydroponically in a controlled greenhouse at a local farmer's factory, and samples of fruit from six stages during ripening, mature green (approximately 35 days after anthesis), breaker (approximately 40 days after anthesis), turning I (orange-yellow, 1 day after breaker), turning II (orange, 2 days after breaker), red (6 days after breaker), and overripe (approximately 12 days after breaker), were harvested.

Seeds of wild-type Rutgers (LA1090) and mutant lines derived from it (LA3001, LA3012, and LA3013) were obtained from the C. M. Rick Tomato Genetics Resource Center (University of California, Davis, CA). All seeds were germinated in pots (500 mL) in a conditioned room, as previously described (*32*). After 3 weeks, all seedlings were moved to 10 L pots containing MagAmpK fertilizer (Hyponex Ltd., ScottsMiracle-Gro) and grown in a greenhouse. The temperature of the greenhouse was maintained at 25 °C under a natural light−dark cycle from February to May. Three plants of each line were grown, and tomato fruits were harvested 50−55 days after anthesis.

Five to seven independent fruits for each tomato line were prepared separately as follows. After the removal of the locular tissue and seeds, the entire fruit of the cherry tomato (cv. Carol 10) was immediately frozen using liquid N_2. Other tomato fruits were cut vertically into eight pieces, and samples represented paired pieces from opposite sides. Locular tissue and seeds were removed, and the pericarp was frozen.

Ethylene Treatment. Tomato (cv. Carol 10) fruits at the mature green stage were used for the experiment. Thirty fruits were treated in a gastight chamber (3.9 L) containing ethylene gas (500 ppm) for 48 h with one replacement of ethylene gas after 24 h. After the ethylene treatment, the samples were removed from the chamber and cut and frozen immediately. Control fruits were treated with air in the same gastight chamber for 48 h with one replacement of air after 24 h.

Extraction of Glycoalkaloids from Tomato Fruits. Each frozen sample was powdered with a mortar and pestle. Powdered samples (100 mg) were extracted with 300 μL of extraction solvent [water containing 5% (v/v) acetic acid and 10 μg/mL genistin as an internal standard]. After samples had been homogenized twice with a Mill MM 300 mixer (Qiagen, Hilden, Germany) at 27 Hz for 2 min, homogenates were centrifuged (12000*g* for 10 min at 4 °C). After the supernatant was removed, an additional 300 μL of extraction solvent was added to the residue, and the extraction was repeated. The pooled extracts (∼600 μL) were filtered through a 0.2 μm PVDF membrane (Whatman, Brentford, U.K.). The filtrate was used for UPLC-TOF-MS analysis. All extracts were stored at −80 °C for no longer than 3 months after extraction. We confirmed that the glycoalkaloids in the extracts were stable for 3 months at −80 °C.

Chemicals. Authentic α-tomatine (**1**) and genistin were purchased from Extrasynthese (Genay, France). Purified esculeoside A (**2**), described previously (*20*), was kindly provided by T. Nohara.

UPLC-TOF-MS Analysis. A Waters UPLC ACQUITY system coupled with a Micromass LCT premier mass spectrometer (Waters Co., Milford, MA) was used for UPLC-TOF-MS analysis. The data were acquired with MassLynx (Waters Co.). The glycoalkaloid extract was applied to a 150 mm × 2.1 mm (inside diameter), 1.7 μm, ACQUITY UPLC BEH-C18 reversed-phase column (Waters Co.). The mobile phase consisted of 0.1% (v/v) aqueous formic acid (solvent A) and 0.1% (v/v) formic acid in acetonitrile (HPLC grade; solvent B). The gradient program was as follows: from 3 to 20% solvent B over 10 min, from 20 to 30% solvent B over 5 min, from 30 to 95% solvent B over 5 min, 95% solvent B for 5 min, and 3% solvent B for 5 min. The flow rate was set to 0.2 mL/min and the column oven temperature to 30 °C. For each sample, 3 μL was injected. To monitor the UPLC chromatogram, a photodiode array detector was used in the wavelength range between 210 and 600 nm. The mass spectra of glycoalkaloids were obtained in positive-ion electrospray ionization (ESI) mode. ESI conditions were optimized using α-tomatine (**1**) and set as follows: capillary voltage of 2.0 kV and sample cone voltage of 185 V. Desolvation and source temperatures were set to 200 and 100 °C, respectively. The desolvation and cone gas (N_2) flows were set at 50 and 750 mL/min, respectively. External mass calibration was applied following the manufacturer's protocol. For quantitative analysis, V mode was selected, and the dynamic range was set to "extended" to maintain mass signal linearity within the dynamic range. Leucine enkephalin was used as a lock mass.

Quantification of α-Tomatine (1) and Esculeoside A (2). The contents of α-tomatine (**1**) and esculeoside A (**2**) were estimated from calibration curves obtained using commercial α-tomatine (**1**) (Extrasynthese) and purified esculeoside A (**2**). The peak areas of *m*/*z* 1034.5 ± 0.3 Da [for α-tomatine (**1**)] and *m*/*z* 1270.6 ± 0.3 Da [for esculeoside A (**2**)] were divided by the area of genistin (internal standard) and used to generate the calibration curves.

LC-FTICR-MS. Liquid chromatography Fourier transform ion cyclotron resonance mass spectrometry (LC-FTICR-MS) using a Finnigan LTQ-FT instrument (Thermo Fisher Scientific, Waltham, MA) coupled with an Agilent 1100 system (Agilent, Palo Alto, CA) was used for estimation of molecular formulas and multistage MS/MS fragmentation of tomato glycoalkaloids. The analytical conditions were the same as those described previously (*32*). Multistage MS/MS analysis was performed with an ion trap detector at a collision energy of 35%.

Statistical Analysis. To determine the contents of α-tomatine (**1**), esculeoside A (**2**), peaks **A**, **B**, and **C** in fruits at different maturing stages, and ripening-inhibition mutants, LC-MS measurement was conducted using three to five independent fruit samples. To determine the contents of α-tomatine (**1**) and esculeoside A (**2**) in the air- and ethylene-treated fruits, LC-MS measurement was conducted using 29

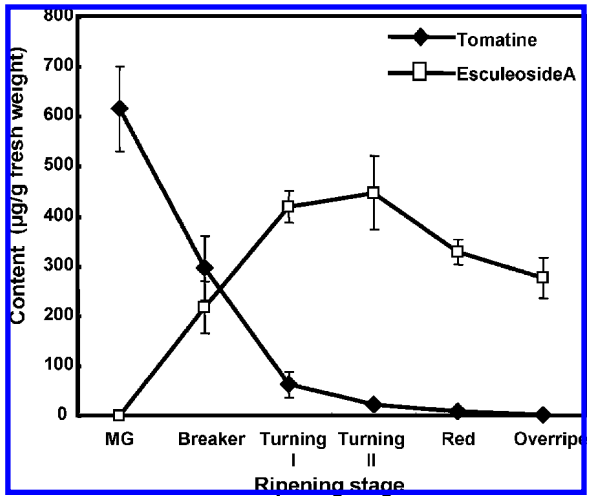

Figure 2. Accumulation levels of α-tomatine (**1**) and esculeoside A (**2**) in mature green (MG), breaker, beginning of turning (Turning I), end of turning (Turning II), red (Red), and overripe stages of Carol 10 fruit. Accumulation levels are given in micrograms per gram of fresh weight of fruit pericarp. Means and standard deviations from measurements of five independent fruits at each stage are given.

and 30 independent fruit samples, respectively. Results are expressed as the mean ± the standard deviation. The statistical significance of differences between means was evaluated with a Student's *t* test using Microsoft Excel 2000.

RESULTS AND DISCUSSION

We first investigated the changes in α-tomatine (**1**) and esculeoside A (**2**) contents during ripening (**Figure 2**). At the mature green stage, esculeoside A (**2**) was undetectable. However, the amount of esculeoside A (**2**) drastically increased from the breaker stage, reaching a maximum level (447 μg/g of fresh weight) at the turning II stage. The esculeoside A (**2**) level then decreased to 277 μg/g of fresh weight at the overripe stage. In contrast, the α-tomatine (**1**) content was highest at the mature green stage (615 μg/g of fresh weight) and decreased with ripening, as previously reported (*5, 14*−*17, 33*). Nohara and co-workers reported that the major component of tomato glycoalkaloids changed with the progression of ripening (*3*). Our result is consistent with this previous finding.

Next, we investigated the effect of exogenous ethylene on glycoalkaloid accumulation using mature green fruits (**Figure 3**). Without the ethylene (control), the α-tomatine (**1**) content stayed at a high level (723 μg/g of fresh weight), and the content of escleoside A (**2**) stayed at a low level (55 μg/g of fresh weight). In the ethylene-treated fruit, however, the α-tomatine (**1**) levels decreased to 251 μg/g of fresh weight, and those of esculeoside A (**2**) rose to 264 μg/g of fresh weight. These results suggest that ethylene promotes an accumulation of esculeoside A (**2**) and a decrease in α-tomatine (**1**) levels.

To confirm the relationship between esculeoside A (**2**) accumulation and ethylene production, we analyzed mutant lines, *rin* and *nor*, each of which produces tomato fruits that fail to ripen due to a lack of increase in ethylene production (*34*). The contents of esculeoside A (**2**) and α-tomatine (**1**) in mutant fruits (50−55 days after anthesis) were compared with those of wild-type Rutgers (**Table 1**). In the wild type, the content of esculeoside A (**2**) was 180.8 μg/g of fresh weight. In contrast, the contents of esculeoside A (**2**) were 1.0 ± 1.7 and 0.3 ± 0.2 μg/g of fresh weight in *rin* and *nor* mutants, respectively (**Table 1**). In *rin* and *nor* fruits, the α-tomatine (**1**) content remained at

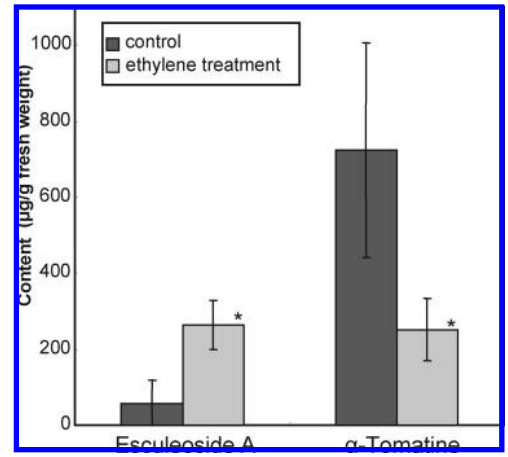

Figure 3. Change in the accumulation levels of α-tomatine (**1**) and esculeoside A (**2**) by exogenous ethylene treatment. Accumulation levels are given in micrograms per gram of fresh weight of fruit pericarp. Black and gray bars depict data for control (untreated) and ethylene-treated samples, respectively. Means and standard deviations were obtained from measurements of 29 (control) and 30 (ethylene treatment) independent fruits. Asterisks denote a significant difference from the control samples in a *t* test ($P < 0.01$).

Table 1. Comparison of Esculeoside A (**2**) and α-Tomatine (**1**) Contents of Wild-Type with Ripening-Inhibition Mutant Lines (cv. Rutgers)

line	mutant	escucleoside A[a] (μg/g of fresh weight)	α-tomatine[a] (μg/g of fresh weight)
LA1090	WT	180.8 ±40.9	5.8 ± 1.7
LA3012	*rin, mc*[b]	1.0 ± 1.7	71.7 ± 22.4
LA3013	*nor*	0.3 ± 0.2	117.5 ± 38.9
LA3001	*Nr*	10.3 ± 6.9	128.4 ± 55.7

[a] Means ± the standard deviation of three to five tomato fruits of each line. [b] LA3012 has the *macrocalyx* (*mc*) mutation, which exhibits a large calyx phenotype, in addition to *rin*.

higher levels, 71.7 and 117.5 μg/g of fresh weight, respectively, than in the wild type (5.8 μg/g of fresh weight). To further demonstrate the relationship between esculeoside A (**2**) accumulation and ethylene perception, we analyzed fruits of the *Nr* mutant. In the *Nr* fruit, the esculeoside A (**2**) content was 10.3 μg/g of fresh weight, whereas the α-tomatine (**1**) content remained at 128.4 μg/g of fresh weight. These results collectively indicate that developmentally regulated ethylene production and ethylene perception are necessary for the accumulation of esculeoside A (**2**).

It has been hypothesized that esculeoside A (**2**) (*m/z* 1270.6 $[M + H]^+$) is formed from α-tomatine (**1**) (*m/z* 1034.6 $[M + H]^+$) by two hydroxylations, one acetylation, and one glycosylation. In cultivar Carol 10, peaks of putative intermediates, *m/z* 1050.5 $[M + H]^+$ (peak **A**), *m/z* 1092.5 $[M + H]^+$ (peak **B**), and *m/z* 1108.5 $[M + H]^+$ (peak **C**), were detected. To confirm the identity of peaks **A**, **B**, and **C** of cultivar Carol 10 with those of other cultivars, we analyzed accurate *m/z* values and multistage MS/MS profiles using LC-FTICR-MS (**Table 2**) and compared the results with the MS data reported previously (*22, 23, 35*−*37*). Peak **A** has been annotated as "singly hydroxylated α-tomatine (**1**)" (*36*). Although the MS/MS fragmentation profile of peak **A** did not match that reported previously probably due to the difference in fragmentation conditions (*36*), multistage MS/MS fragments were consistently assigned to singly hydroxylated α-tomatine (**1**). The accurate

Table 2. Accurate *m/z* Values of Quasi-Molecular Ions and Multistage MS/MS Data of Peaks **A**, **B**, and **C** and Esculeoside A (**2**) by LC-FTICR-MS Analysis

peak	detected *m/z* $[M + H]^+$	molecular formula[a]	predicted metabolite	target ion	MS^n	product ions [relative abundance (%)]
A	1050.54709	$C_{50}H_{83}NO_{22}$	singly hydroxylated α-tomatine (**1**) (*36*)	1050.5	MS^2	1032.5 (100), 414.4 (3)
				1032.5	MS^3	900.5 (100), 414.4 (69), 576.5 (37), 1014.5 (27), 1003.4 (12)
				414.4	MS^4	273.2 (100), 396.4 (37), 385.1 (31), 255.1 (8)
B	1092.55837	$C_{52}H_{85}NO_{23}$	lycoperoside A, B, or C (*18*)	1092.5	MS^2	1032.5 (100), 1074.5 (7), 474.4 (2), 414.4 (2)
				1032.5	MS^3	414.4 (100), 900.5 (68), 1003.4 (53), 1014.5 (53), 576.5 (48)
				414.4	MS^4	273.2 (100), 396.4 (69), 385.1 (57), 255.1 (45)
C	1108.55418	$C_{52}H_{85}NO_{24}$	esculeoside A lacking *O*-27 glucosyl residue (*36*)	1108.5	MS^2	1048.5 (100), 1090.4 (10), 1030.4 (5), 412.3 (3)
				1048.5	MS^3	430.3 (100), 1001.4 (93), 916.4 (41), 592.4 (37), 1030.5 (32), 412.4 (20)
				430.3	MS^4	383.3 (100), 273.2 (33), 412.4 (22), 255.2 (21), 396.4 (17)
esculeoside A (**2**)	1270.60663	$C_{58}H_{95}NO_{29}$		1270.6	MS^2	1210.5 (100), 1048.5 (7.8), 1030.4 (6), 592.4 (5)
				1210.5	MS^3	1048.5 (100), 592.4 (23), 754.5 (17), 430.3 (7)
				1048.5	MS^4	1001.4 (100), 430.4 (75), 916.4 (67), 592.4 (47), 1030.5 (38)
				430.4	MS^5	383.3 (100), 273.2 (72), 412.4 (54), 255.1 (26), 396.4 (3)

[a] Estimated from detected accurate *m/z* values.

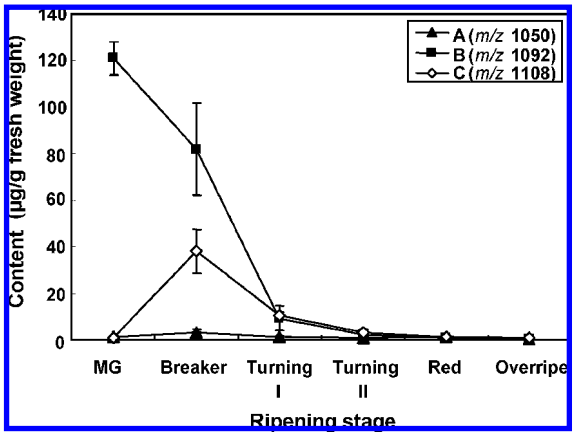

Figure 4. Accumulation of peaks **A**, **B**, and **C** at mature green (MG), breaker, beginning of turning (Turning I), end of turning (Turning II), red (Red), and overripe stages of Carol 10. Accumulation levels are given in micrograms per gram of fresh weight of fruit pericarp. The amounts of each compound were calculated from the area of quasi-molecular ion using a calibration curve for α-tomatine. Means and standard deviations from measurements of five independent fruits at each stage are given.

m/z value and multistage MS/MS profile of peak **B** (**Table 2**) suggested that peak **B** is identical to lycoperoside A, B, or C [structures determined previously (*18*)]. Peak **C** has been annotated as "esculeoside A lacking *O*-27 glucosyl residue" (*36*). We obtained results consistent with this annotation. Accurate *m/z* value and multistage MS/MS data (**Table 2**) suggested that the hexose moiety present in esculeoside A (**2**) is lacking in peak **C**, and that aglycone part of esculeoside A (**2**) was identical to peak **C**. Although the steric configurations of these compounds still need to be clarified, these results suggest that peaks **A**, **B**, and **C** are intermediates of synthesis of esculeoside A (**2**) from α-tomatine (**1**).

We next investigated changes in the contents of these compounds during ripening of Carol 10 fruit (**Figure 4**). The contents were estimated using a calibration curve for α-tomatine (**1**), assuming that their response factors were similar. Peak **A** accumulated at trace levels at the mature green stage and disappeared after the turning stage. The content of peak **B** was the highest among the three compounds, accumulating to approximately 20% of the level of α-tomatine (**1**) at the mature green stage. Levels then gradually decreased during ripening and reached trace levels at the red and overripe stages. Peak **C** was not detected at the mature green stage but showed the highest accumulation level at the breaker stage (38 μg/g of fresh weight); it then decreased after the turning stage. The transient accumulation patterns of peaks **A**, **B**, and **C** suggest that they are intermediates of esculeoside A (**2**) biosynthesis. We then compared the amounts of peaks **A**, **B**, and **C** in fruits of ripening mutants to those in wild-type Rutgers fruit. In *rin*, *nor*, and *Nr*, the three compounds, particularly peak **B**, accumulated to higher levels than in the wild type (**Table 3**). This result, together with the remarkable decrease in the level of esculeoside A (**2**) in the ripening mutant fruits (**Table 1**), demonstrates that conversion of peak **C** to esculeoside A (**2**) is an ethylene-dependent step.

Table 3. Contents of Metabolites Corresponding to Peaks **A**, **B**, and **C** in Wild-Type and Ripening-Inhibition Mutant Lines (cv. Rutgers)

line	mutant	peak **A**,[a] *m/z* 1050.5 (μg/g of fresh weight)[b]	peak **B**,[a] *m/z* 1092.5 (μg/g of fresh weight)[b]	peak **C**,[a] *m/z* 1108.5 (μg/g of fresh weight)[b]
LA1090	WT	0.8 ± 0.6	0.8 ± 0.7	0.7 ± 0.8
LA3012	*rin*, *mc*[c]	6.8 ± 1.1	76.7 ± 16.8	8.4 ± 5.9
LA3013	*nor*	15.1 ± 0.1	131.4 ± 27.4	11.1 ± 2.4
LA3001	*Nr*	8.8 ± 6.1	130.1 ± 61.7	35.5 ± 21.0

[a] Means ± the standard deviation of three to five tomato fruits of each line. [b] Amount determined by using a calibration curve of α-tomatine. [c] LA3012 has the *macrocalyx* (*mc*) mutation, which exhibits a large calyx phenotype, in addition to *rin*.

In addition to the intermediates, we also detected an esculeoside A-hexose that undergoes an addition of one hexose moiety (*m/z* 1432.65890 $[M + H]^+$, $C_{64}H_{105}NO_{34}$). It was also reported that degradation products of esculeoside A (**2**), such as 3β-hydroxy-5α-pregn-16-en-20-one and its lycotetraoside, are present in overripe fruit (*38*, *39*). These results demonstrate that esculeoside A (**2**) is not a final product; rather, it undergoes further modification and degradation during ripening.

In this study, we demonstrated the putative metabolic linkage between α-tomatine (**1**) and esculeoside A (**2**) and the involvement of ethylene in esculeoside A (**2**) biosynthesis during tomato fruit ripening. We need to mention that further study using a radiolabeled compound will be needed to unequivocally demonstrate that esculeoside A (**2**) is biosynthesized from α-tomatine (**1**). It is noteworthy that esculeoside A (**2**) inhibits the growth of human breast cancer MCF7 and mouse melanoma B16F2F cells (*20, 21*), and its aglycone, esculeogenin A, reduces atherogenesis by suppressing the activity of acyl-CoA:cholesterol acyltransferase (*40*). Thus, understanding the mechanisms that control esculeoside A (**2**) accumulation will provide a basis for improving the dietary value of red ripe tomato fruit as a source of potentially health-beneficial compounds.

ABBREVIATIONS USED

rin, *ripening-inhibitor*; *nor*, *nonripening*; *Nr*, *never-ripe*; UPLC-TOF-MS, ultraperformance liquid chromatography time-of-flight mass spectrometry; LC-FTICR-MS, liquid chromatography Fourier transform ion cyclotron resonance mass spectrometry.

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Supporting Information Available: A figure illustrating fragmentation schemes of peaks **A**, **B**, and **C** and esculeoside A (**2**) in which major fragments shown in **Table 2** are assigned. This material is available free of charge via the Internet at http://pubs.acs.org.

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