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Mass Spectrometry Imaging Analysis of Metabolic Changes in Green and Red Tomato Fruits Exposed to Drought Stress

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Abstract: Plant metabolism is altered in response to various environmental changes. In vegetable crops such as tomato (*Solanum lycopersicum*), the metabolic composition of fruits varies depending on the variety or cultivar as well as the cultivation method used. Few studies have examined the metabolic fluctuations in fruits under stress conditions, such as drought. We previously examined the metabolomes of mature green tomato fruits, which undergo drastic changes in chemical composition during ripening, and mature red fruits in response to drought stress. We detected or predicted fluctuations in the levels of fatty acids and phospholipid constituents, such as inositol and ethanolamine. In this study, we determined the localizations of these metabolites in fruits using mass spectrometry imaging. The accumulation patterns of stearic acid and palmitic acid were similar, but unlike these fatty acids, oleic acid accumulated to high levels in the placenta. Inositol is involved in various physiological processes; under drought conditions, this metabolite is synthesized by a different pathway compared to under normal conditions. The biosynthesis of pectin, a component of the gel surrounding the seeds, was suppressed under drought stress but increased in seeds. We propose that under drought conditions, a shift to phospholipid biosynthesis occurs that protects seeds from dehydration.

Keywords: tomato; drought stress; metabolome; transcriptome; mass spectrometry imaging; cell membrane; fatty acid; phospholipid; cell wall

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1. Introduction

Tomato (*Solanum lycopersicum*) is a popular and nutritious crop, with the fruits containing high levels of vitamin C, potassium, beta carotene, and lycopene [1,2], as well as functional metabolites such as GABA and tomatine. Approximately 8000 tomato cultivars are grown globally, and novel cultivars continue to be created [3,4]. As tomatoes are eaten as whole fruits, including the pericarp, the hardness of the pericarp affects the eating quality of the tomato fruit. When plants are exposed to drought, they synthesize compatible solutes (also known as osmoprotectants) that prevent transpiration [5,6]. Apart from functioning in drought tolerance, these solutes affect fruit texture [5]. In vegetable crops such as tomato, the metabolic composition of fruits changes in response to drought or salt stress during fruit development [3,7]. In addition, drought stress affects the ripening period of fruits and the number of fruits produced [8].

We previously examined the transcriptomes and metabolomes of green mature tomato fruit (maximum size but immature) and mature, red fruits under drought stress. We

identified genes related to fatty acid biosynthesis and the resulting fatty acids, whose expression increased in the drought-stressed group compared to the control. Furthermore, cell wall- and cell membrane-related genes were upregulated by drought stress. These findings suggest that the biosynthesis of phospholipids in cell membranes increases in response to drought stress [9].

Metabolome analysis is an excellent method for analyzing the chemical composition of fruits over time. However, although it is possible to measure the total content of each metabolite in a sample, metabolome analysis cannot be used to map the distribution of these metabolites. To examine the distribution of each metabolite using metabolome analysis, the samples must be dissected and the metabolites in each region of the fruit must be quantified individually. However, mass spectrometry imaging (MSI) can be used to visualize the localization of each metabolite of interest in the fruit and to analyze multiple metabolites simultaneously, revealing which metabolites co-accumulate under certain conditions [10–14]. In this study, we used MSI to analyze the stage-specific localization of metabolites whose levels fluctuate in drought-stressed tomato fruits. Phosphatidylcholine is the most abundant phospholipid in plant cells, followed by phosphatidylethanolamine and phosphatidylinositol [14]. The ratio of unsaturated to saturated fatty acids increases during freezing stress [15]. Here, we focused on inositol, whose level was shown to increase in drought-stressed tomato fruit, as detected by metabolome analysis, and investigated the localizations of inositol, phosphatidyl inositol, oleic acid, and stearic acid by MSI. We also analyzed β-alanine and tomatidine accumulation in tomato fruits during development and under drought stress by MSI. Analyzing the localizations of various metabolites in tomato fruits by MSI is an excellent method for measuring their accumulation and distribution in the fruits. Our findings lay the foundation for breeding tomato fruit with improved drought tolerance and for profiling fruit metabolites in other crops threatened by drought stress.

2. Materials and Methods

2.1. Samples and Cultivation

The tomato (*Solanum lycopersicum*) cultivar Micro-Tom, an experimental dwarf tomato variety, was used in this study. The seeds were obtained from the National BioResource Project (NBRP) at the University of Tsukuba Gene Experiment Center, Japan. The plants were cultivated in a greenhouse with a controlled temperature of 25 °C for 12 h during the day and 20 °C for 12 h at night. For cultivation, Hyuga soil was soaked with sufficient amounts of water, and the seeds were sown in a groove ~1 cm deep and covered with clay. The seedlings were fed with liquid fertilizer (Sumitomo Chemical Co., Ltd., Nihombashi, Chuo-ku, Tokyo, Japan) and diluted 500× until they developed one or two true leaves. The seedlings in Hyuga soil were transferred to 15 cm-diameter plastic pots (within 8 min) and allowed to grow. Only distilled water was provided until the plants flowered. After flowering, the control (C) plot was supplemented with liquid fertilizer (diluted 500×) on the first day and watered the following day. This cycle was repeated until cultivation was completed. The drought-stressed (S) plot was provided with liquid fertilizer (diluted 500×) only once every 7 days. This cycle was repeated until cultivation was completed. The leaves of the drought-stressed plants were slightly rounded and tended to wilt. The fruits reached the mature green stage ~30 days after flowering and the mature red stage ~40 days after flowering. Both stages of fruits from plants grown under S and C conditions were harvested (Figure 1). The fruits were freeze-dried in liquid nitrogen immediately after collection and stored in a deep freezer at −80 °C.

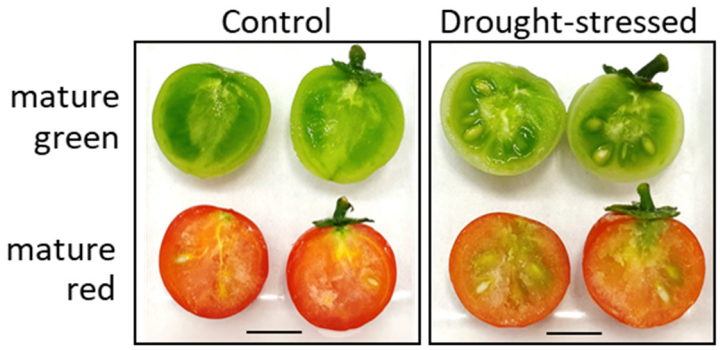

Figure 1. Photographs of the tomato fruits used in this study. Mature green fruits and mature red fruits were subjected to mass spectrometry imaging. All tomato fruits were harvested at 10 a.m. The scale bar indicates 1 cm.

2.2. Mass Spectrometry Imaging

The frozen fruit samples were embedded in cryo-embedding medium and cut into serial sections (14 μm) using a cryostat (NX70; Thermo Fisher Scientific, 168 third Avenue Waltham, MA, USA) [11]. The sections were sprayed with a matrix of α-cyano-4-hydroxycinnamic acid (CHCA)-acetonitrile/water/TFA = 50:49.9:0.1 using a TM sprayer (HTX Tech., Chapel Hill, NA, USA). Matrix-assisted laser desorption/ionization (MALDI) mass spectra were acquired using a MALDI-TOF mass spectrometer (rapifleX; Bruker Daltonik GmbH, Wien, Austria) equipped with an Nd: YAG laser [4]. The sections were scanned and laser spot areas (200 shots) were detected with a spot-to-spot center distance (100 μm) in each direction of the tomato sample. Signals between m/z 150 and 1000 were collected. The surface of each section was irradiated with YAG laser beams in positive ion detection mode. The resulting MS spectra were reconstructed into an MS image with a mass bin width of $m/z \pm 0.1$ from the exact mass using FlexImaging 4.0 software (Bruker Daltonik GmbH, Wien, Austria). The peak intensity value of the spectra was normalized by dividing the value by the total ion current (TIC) to achieve a semi-quantitative analysis between the S and C samples. Optical images of the sections were obtained using a Canon scanner (GT-X820; Canon, Tokyo, Japan), followed by MALDI-TOF imaging of the section.

2.3. Transcriptome and Metabolome Data

The transcriptome data for mature green tomato fruits were obtained from the Gene Expression Omnibus database at the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/geo/ (accessed on 23 October 2019); GEO Series ID GSE139290). The metabolome data were obtained from our previous report [9].

3. Results

3.1. Mass Spectrometry Imaging

3.1.1. Fatty Acids

We analyzed the contents of palmitic acid, stearic acid, and oleic acid in red and green tomato fruit grown on plants subjected or not to drought stress. The pattern of palmitic acid accumulation in the drought-stressed group was different from that of the control (Figure 2). During the ripening period, palmitic acid accumulated throughout the fruit in the drought-stressed group, especially in the outer pericarp and seeds (Figure 2(b2)). During the mature period, palmitic acid levels in the seeds increased significantly, and high levels were also observed in the gel surrounding the seeds in the drought-stressed group (Figure 2(b4)). The changes in the levels and localization of stearic acid (Figure 2c) were highly similar to those of palmitic acid.

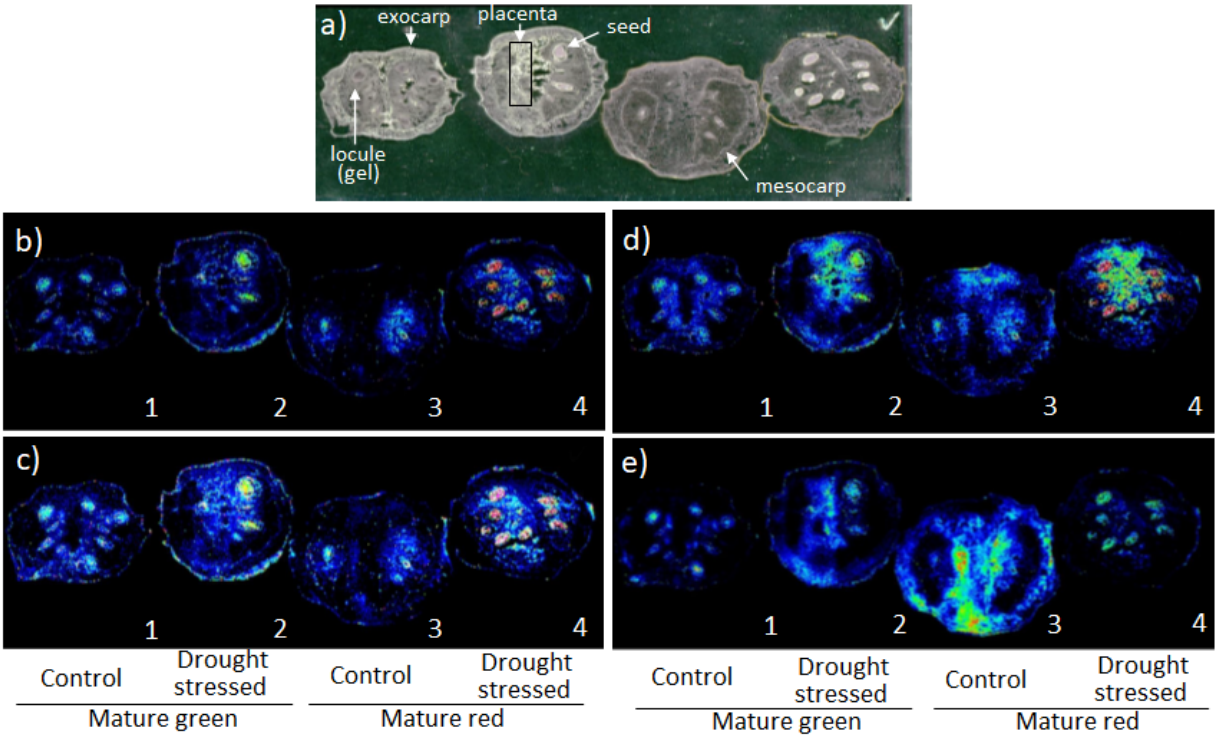

Figure 2. Mass spectrometry imaging of fatty acid and inositol distribution during ripening in tomato fruits under drought stress. (**a**) Bright-field images of control or drought-stressed fruits. (**b**–**d**) Mass spectrometry imaging of palmitic acid +H m/z 257 (**b**), stearic acid +H m/z 285 (**c**), oleic acid +H m/z 283 (**d**), and inositol +Na m/z 203 (**e**). Red pixels represent the highest signal intensity (100%) of the particular ion, and black pixels represent the lowest signal (0%). For interpretation of the references to color in this figure legend, the reader should refer to the web version of this article.

Oleic acid was localized to the seeds and gels surrounding the seeds over a wide region during the ripening period, especially in the control group (Figure 2(d1)). In the drought-stressed group, oleic acid was detected in the placenta and outer pericarp of the fruit (Figure 2(d2)). During the mature stage, this metabolite was observed in the seeds and gel around the seeds in the control group (Figure 2(d3)), whereas very high levels were detected in the seeds and placenta in the drought-stressed group (Figure 2(d4)). The localization patterns of oleic acid in the ripening and mature stages were similar, and this metabolite accumulated in the placenta when drought stress was applied. Furthermore, very high levels of oleic acid were detected in the seeds of mature fruits in the drought-stressed group (Figure 2(d4)).

3.1.2. Inositol

The localization of inositol (Figure 2e) was strongly affected by drought stress during the ripening period. In addition, almost no inositol was observed in the gel surrounding seeds throughout the maturation process.

During the ripening period, inositol levels increased overall in the drought-stressed group, and this metabolite was detected in seeds, the middle pericarp, and the outer pericarp Figure 2(e2). In mature fruits, high levels of inositol were detected in the mesocarp in the control group (Figure 2(e3)). However, in the drought-stressed group, almost no inositol was detected in the mesocarp, but strong signals were detected in the seeds and the gel around the seeds (Figure 2(e4)). Based on the imaging results, under normal conditions, inositol accumulates in seeds during the early stages of development, and its localization changes to the mesocarp as maturation progresses. However, in the presence of drought stress, inositol began to accumulate in the mesocarp during the early stage of fruit development and was concentrated in seeds during the full maturity period (mature red).

3.1.3. β-Alanine

Throughout the ripening and mature periods, β-alanine accumulated markedly in the gel around the seeds, whereas no β-alanine was observed in the seeds. During ripening, drought stress increased the amount of β-alanine accumulation in the gel around the seeds. By contrast, in the mature fruits, there was no significant difference between the amount of β-alanine accumulation in the gel around the seeds in the control and drought-stressed groups(Figure 3b).

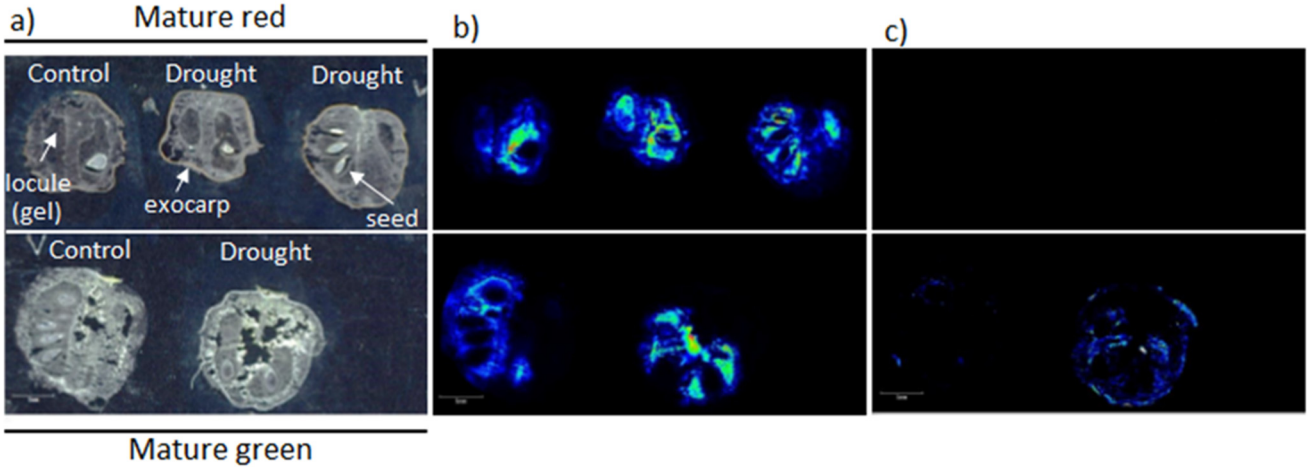

Figure 3. Mass spectrometry imaging of functional ingredients in tomato fruits during ripening under drought stress. (**a**) Bright-field images of control or drought-stressed fruits. (**b**,**c**) Mass spectrometry imaging of β-Alanine +Na *m*/*z* 112 (**b**) and tomatidine +H *m*/*z* 416 (**c**).

3.1.4. Tomatidine

Tomatidine accumulation was observed in the fruits during the ripening period, but not in the mature fruits. During ripening, tomatidine was detected throughout the outer pericarp and gel in the drought-stressed group (Figure 3c).

4. Discussion

Here, we discuss the metabolic changes detected in tomato fruit subjected to drought stress and compare our results, obtained using MSI, with previously published transcriptome and metabolome data. Based on these observations, the metabolic pathways predicted to be induced by drought stress are shown in Figure 4.

4.1. Fatty Acid and Phospholipid Biosynthesis

Stearic acid, palmitic acid, and oleic acid are constituents of phosphatidylcholine, phosphatidylinositol, and phosphatidylethanolamine [14]. Metabolome analysis revealed an increase in inositol and 2-ethanolamine levels in tomato fruits under drought stress, suggesting that under these conditions, fatty acid biosynthesis is activated, along with phospholipid biosynthesis.

Palmitic acid, stearic acid, and oleic acid are used to synthesize phosphatidic acid (PA) via conjugation to glycerol 3P through acyl-bond formation [14]. The PA pathway separates into the 1,2-Diacyl-sn-glycerol or CDP-1,2-Diacyl-sn-glycerol pathway: the 1,2-Diacyl-sn-glycerol pathway synthesizes phosphatidylcholine and phosphatidylethanolamine, and the CDP-1,2-Diacyl-sn-glycerol pathway synthesizes phosphatidylinositol [14].

The levels of 2-ethanolamine and inositol in the ripened fruits increased approximately 2.7-fold compared to the control group. The metabolism of 2-ethanolamine generates two metabolites: phosphoethanolamine and CDP-ethanolamine. These metabolites are metabolized to phosphatidylcholine or phosphatidylethanolamine, respectively [14].

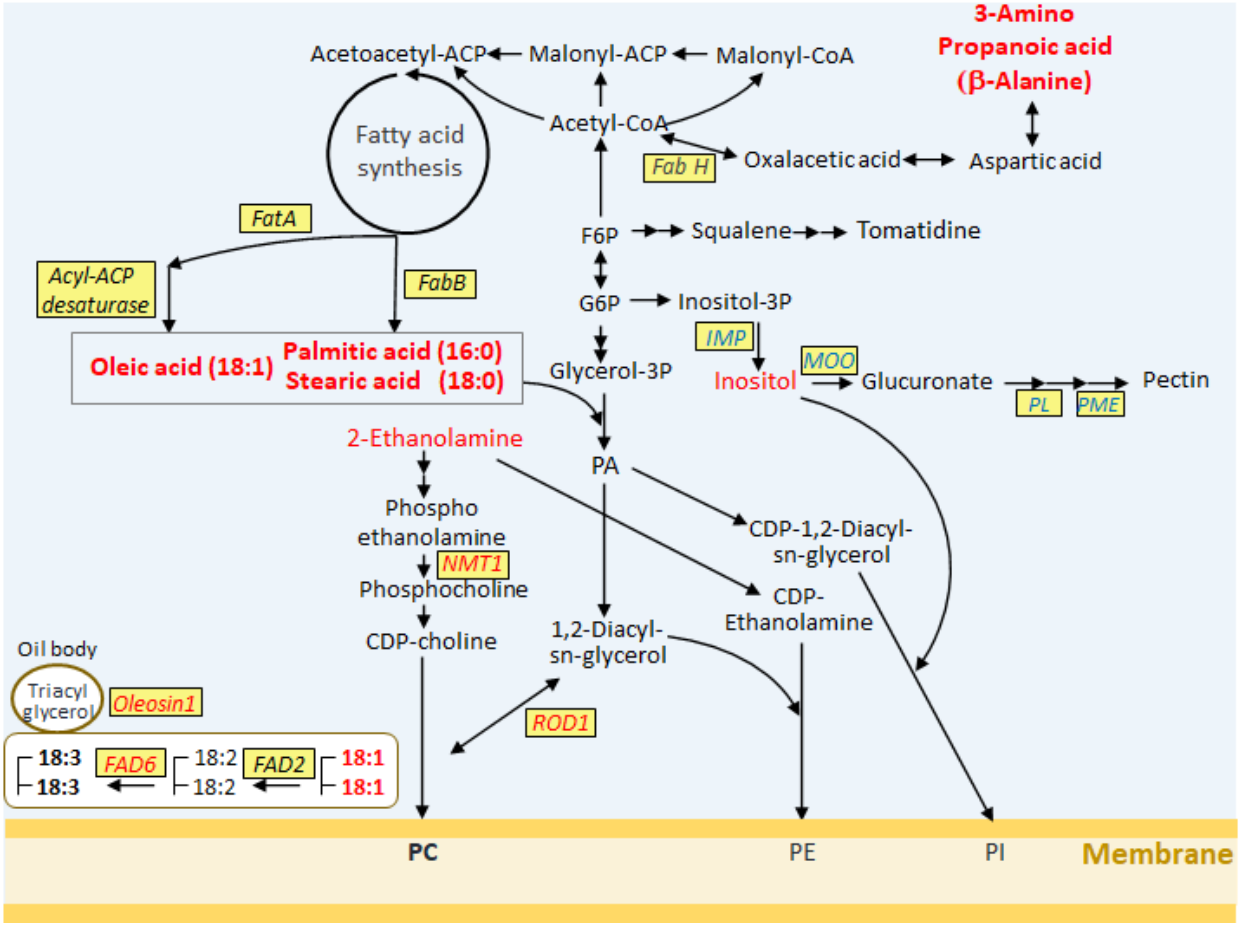

Figure 4. Model of the metabolic pathways in tomato fruits under drought stress. Genes or components upregulated or downregulated by drought-stressed conditions are shown in red and blue, respectively. The enzyme-encoding genes that were extracted as differentially expressed genes (DEGs) based on enrichment analysis of microarray data are shown in italics. The metabolites identified as accumulating by MSI are marked by bold font. The reaction in the white box shows desaturation of the PC constituent fatty acids in the ER. G6P, Gluctose-6-phosphate; F6P, Fluctose-6-phosphate; PA, phosphatidic acid; FabB, 3-oxoacyl-(acyl-carrier-protein) synthase I, chloroplastic; FabH, 3-oxoacyl-ACP synthase3; FatA, oleoyl-acyl carrier protein thioesterase, chloroplastic; NM,; phosphoethanolamine N-methyltransferase; ROD1, phosphatidylcholine:diacylglycerolcholine phosphotransferase 1; IMP, inositol monophosphatase; MOO, inositol oxygenase; PME, pectin esterase; PL, pectate lyase; FAD2, delta-12 fatty acid desaturase; FAD6, omega-6 fatty acid desaturase.

The level of phosphoethanolamine N-methyltransferase 1 (NMT1), which converts phosphoethanolamine to phosphocholine, increased in the drought-stressed group versus control group [9].

We previously determined that phosphatidylcholine and dipalmitoyl-phosphatidylcholine (DPPC) levels increased with the activation of fatty acid biosynthesis in tomato fruits at the mature stage, especially in seeds and the gels around seeds [9]. We also detected an increase in the levels of fatty acids, ethanolamine, and inositol, suggesting that the accumulation of phosphatidylinositol and phosphatidylethanolamine increases under drought stress. Based on these findings, phospholipid levels must also increase in tomato fruits during the ripening period under drought conditions.

Phospholipids likely accumulate in the outer pericarp to prevent water loss during drought stress. In addition, seeds are likely protected from dehydration by accumulating phospholipids during the mature period.

4.2. Triacylglycerol Accumulation

When fatty acid biosynthesis is activated and PA biosynthesis is upregulated, subsequent metabolic steps lead to the accumulation of triacylglycerols [16]. An increase in the expression of phosphatidylcholine: diacylglycerol cholinephosphotransferase 1 (ROD1) was observed during fruit ripening. This enzyme transfers the fatty acid moiety of phosphatidylcholine into the fatty acid moiety of 1,2-Diacyl-sn-glycerol [14]. The desaturation of fatty acids composed of phosphatidyl choline occurs in the ER. Delta-12-fattyacid desaturase (FAD2) is responsible for the desaturation of oleic acid into linoleic acid. Linoleic acid is then converted to a-linolenic acid by omega-6 fatty acid desaturase (FAD6) (Figure 4). ROD1 changes unsaturated fatty acids composed of PC to saturated fatty acids composed of 1,2-Diacyl-sn-glycerol [14]. By repeating this reaction, the levels of unsaturated fatty acids composed of triacylglycerol increase, and triacylglycerol accumulates in oil bodies in seeds [16]. Oil bodies contain the protein oleosins. Transcriptome analysis revealed an increased in expression of the gene encoding Oleosin1, which accumulates in seeds along with triacylglycerol. The accumulation of oil bodies in seeds might help protect the seeds from dehydration.

4.3. Inositol Biosynthesis

The main metabolic pathway generating inositol involves the dephosphorylation of inositol-3P by inositol mono phosphatase (IMP) starting from G6P [17–20]. Our IMP analysis revealed a marked change in inositol localization in the tomato fruits, especially during the mature period. In the control group, high levels of inositol were detected in the middle pericarp, but in the drought-stressed group, inositol levels were high in seeds.

On the other hand, the transcript levels of IMP and inositol oxygenase (MOO) genes decreased under drought stress (Supplementary Table S1 and Figure S1). After being synthesized by IMP, inositol is metabolized by MOO to glucuronate and then to pectin [21]. Transcriptome analysis showed that the expression levels of genes encoding multiple enzymes involved in pectin biosynthesis were reduced by drought stress [9].

These results suggest that inositol is expressed in the mesocarp under normal conditions and that inositol is involved in pectin synthesis. However, under drought stress, inositol biosynthesis via IMP is suppressed and pectin biosynthesis is also downregulated. On the other hand, inositol binds to 1,2-Diacyl-sn-glycerol, is metabolized to phospholipids as phosphatidylinositol, and accumulates in seeds [8]. Inositol might be used as a source of phospholipids to protect seeds from dehydration rather than for pectin biosynthesis in the mesocarp and outer pericarp.

4.4. Alanine and Tomatidine

Tomato fruit is rich in functional components. The accumulation of components that improve brain function in humans, such as β-alanine and GABA, increases in tomato fruits in response to drought stress. [22]. Tomatidine, a glycoside found in green tomatoes, might help prevent depressive symptoms in humans [2,22]. In addition, β-alanine levels significantly increase in dried tomato fruits [9]. As shown in Figure 3, β-alanine did not accumulate in seeds, unlike the above-mentioned phospholipids. β-alanine accumulation markedly increased during the ripening period in the drought-stressed group. On the other hand, although tomatidine accumulated during the ripening period, it was not detected during the mature period.

Both β-alanine and tomatidine showed marked changes in accumulation in response to stress response during the ripening period. Since tomatidine is subsequently metabolized to tomatine, these changes might also affect the amount of tomatine that accumulates in tomato fruits.

The finding that drought stress induces the accumulation of fruit-specific functional components, such as tomatidine, is quite important in terms of breeding and food chemistry. This information lays the foundation for future research.

As described above, using MSI, we successfully observed changes in the expression and localization of specific metabolites in tomato fruits during ripening and in response to drought stress. By connecting the changes in the expression and localization of various substances, it is possible to estimate the changes in the associated metabolic pathways.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app12010216/s1. Figure S1: Comparison of MOO transcript levels in tomato fruits by RT-qPCR. Table S1: Primers used for RT-qPCR. The total RNA extraction was conducted with reference to Asakura et al. [9]. For RT-qPCR analysis, cDNA was synthesized from 1 µg of total RNA using SuperScript IV Reverse Transcriptase (Thermo Fisher Scientific) according to the manufacturer's instructions. PowerUp SYBR Green Master Mix (Thermo Fisher Scientific) was used with an ABI 7500 real-time PCR system (Thermo Fisher Scientific). The thermal cycling program was as follows: denaturation at 95 °C for 2 min and 40 amplification cycles at 95 °C for 15 s, followed by 60 °C for 1 min. Melting curves were constructed after the 40 amplification cycles to confirm the specificity of the reactions. The 2–ΔΔCT method was used to calculate the relative expression levels of five genes, following normalization to EF1a (LOC 544055), which was previously used as a reference gene in tomato fruit [9]. The primer sequences are shown in Supplementary Table S1.

Author Contributions: H.A. and S.T. designed and carried out the experiments. H.A., J.F., T.Y. and T.A. wrote the paper. H.A., S.T. and T.A. interpreted the data. T.A. and K.A. conceived and supervised this research. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The microarray data are available in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/ (accessed on 23 October 2019); GEO Series ID GSE139290).

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Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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