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Note

Dynamic metabolic changes during fruit maturation in *Jatropha curcas* L.

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Abstract To understand how metabolism changes during fruit ripening in *Jatropha curcas* L., we performed a non-targeted analysis of metabolites in fruit (the pericarp and developing young seeds) from each maturation stage by means of liquid chromatography-Orbitrap-mass spectrometry, which provides *m*/*z* data with approximately 2 ppm precision. The chromatographic data were processed using bioinformatics tools. The total number of metabolites detected decreased substantially with fruit maturation. Self-organizing map (SOM) analysis and metabolite annotation of the ions detected suggested that dynamic metabolic changes occur during fruit maturation. All chromatographic data were deposited in databases accessible by the public.

Key words: Comparative metabolome analysis, fruit maturation, liquid chromatography-Orbitrap-mass spectrometry.

Jatropha curcas L., a perennial deciduous shrub belonging to the family Euphorbiaceae, has been utilized historically for hedging and in green manure and fertilizers, food, soaps, pesticides, charcoal, and traditional medicine. More recently, the oil of the seed has attracted attention as a source material for the production of biodiesel fuels (Kumar and Sharma 2008; Devappa et al. 2010). To further the potential industrial and medicinal uses of Jatropha, several groups have conducted extensive chemical analyses of the metabolites present in various parts of the plant, such as seeds, leaves, roots, and stems (Martínez-Herrera et al. 2006; Goel et al. 2007). Because Jatropha contains potential cocarcinogens such as phorbol esters, it is crucial that Jatropha metabolites be fully characterized so that the plant may be used safely. However, little is known about the variations of metabolites during fruit maturation.

Over the past decade, the use of metabolomics techniques to dissect plant metabolism has increased dramatically (Hall 2011). Fruit metabolites in strawberry (Aharoni et al. 2002) and tomato (Iijima et al. 2008) have been analyzed through non-targeted approaches involving Fourier transform ion cyclotron resonance mass spectrometry (FT/ICR-MS) and liquid chromatography coupled with FT/ICR-MS, respectively. Both of these methods provide *m*/*z* data with the precision of a few ppm, permitting determination of chemical formulae. The infusion method used by Aharoni et al. (2002) revealed at least 5,000 metabolites in the fruit of strawberry, while the tomato metabolite annotation study of Iijima et al. (2008) identified 850 metabolites produced during fruit maturation. These results demonstrate that metabolite annotation based on precise *m*/*z* data enables the extensive analysis of metabolite dynamics during various plant biological processes, such as fruit maturation. No such metabolomics approach utilizing precise *m*/*z* data, to our knowledge, has been carried out for Jatropha biology.

In this study, we analyzed the metabolites of maturing stages of Jatropha fruit by means of liquid chromatography coupled with Orbitrap mass spectrometry (LC-Orbitrap-MS), which provides *m*/*z* data with 2 ppm precision. The chromatograms were analyzed using bioinformatics approaches, and the resulting data indicate that dynamic metabolic changes occur during fruit maturation in Jatropha.

Jatropha plants were grown in an open experimental field at the University of the Ryukyus (Okinawa island, Okinawa, Japan), located in a subtropical area. Fruit from 4 stages of maturation (2-weeks after flowering (WAF)

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(stage 1), 3-WAF (stage 2), 5-WAF (stage 3), and 10-WAF (stage 4)) was harvested (Figure 1), immediately frozen in liquid nitrogen, and then kept at −80°C. Before extraction of metabolites from the pericarp, hardened drupaceous parts (the endocarp including seeds) of the stage 4 fruits were excised. The frozen samples were homogenized into powder with a mortar and pestle, and then 200 mg of the powdered fruit (in triplicate for each stage) was mixed with 600 µl of 70% methanol containing 25 µM 7-hydroxy-5-methylflavone as an internal standard. After homogenization using a Mixer Mill MM300 disruptor (QIAGEN, Valencia, CA, USA) set at 25 Hz for 2 min twice, homogenates were centrifuged (20000 ***g***, 5 min, 4°C). The supernatant was filtered through a 0.2 µm pore-membrane filter (Millex-LG, 4 mm i.d. disk, Millipore, Bedford, MA, USA).

Mass spectrometry was carried out as described by Iijima et al. (2008), using an LC-Orbitrap-MS (Ion trap Orbitrap XL, Thermo Fisher Scientific Inc., San Jose, CA, USA) with electrospray ionization (ESI) instead of an LC-FT/ICR-MS. Briefly, lidocaine, prochloraz, reserpine, and bombesin were incorporated into the LC solvent as internal standards for positive ion mode analyses. Accurate mass values were calibrated based upon the internal standards, and the exact *m*/*z* values of detected ions were determined with a precision of approximately 2 ppm. Each sample was analyzed over the *m*/*z* range 100–1,500.

Interestingly, the total number of metabolite ions detected under the analytical conditions described above was significantly lower in the more mature fruits (Figure 2). As the same amount of each fruit stage (based on fresh weight) was analyzed, these results could be interpreted to represent the actual metabolic changes occurring during fruit maturation.

To analyze the metabolite dynamics in detail, the raw data from 12 chromatograms were processed computationally. All of the raw binary chromatographic files generated by the LC-Orbitrap-MS were converted into text format and then the ion peaks were extracted using the program PowerFT (http://www.kazusa.or.jp/komics/software/PowerFT/). The 12 resulting PowerFT output files were then analyzed using IonMatch, which generated an aligned matrix of identical ions found in multiple chromatograms based on exact mass value, MS/MS fragmentation pattern (if available), and ion retention time. Possible false alignments in the matrix were detected using the program MatchedIonsFinder (Yamamoto et al. 2012; http://www.kazusa.or.jp/komics/software/MatchedIonsFinder/index.html). Possible chemical formulae that could match the detected ions were calculated using the subprogram

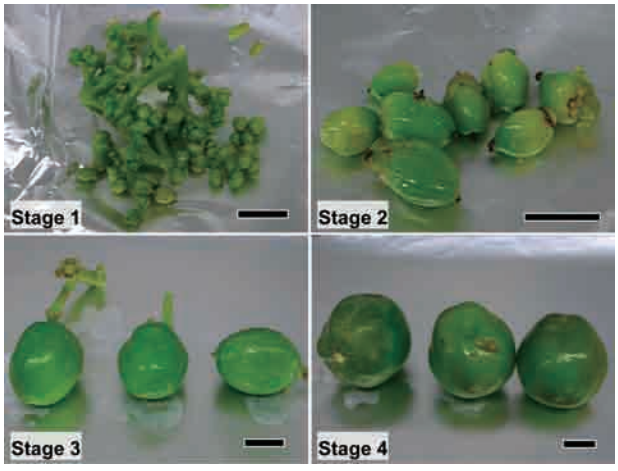

Figure 1. The maturation stages of Jatropha fruits used for metabolite extraction in the present study. Scale bar denotes 1 cm.

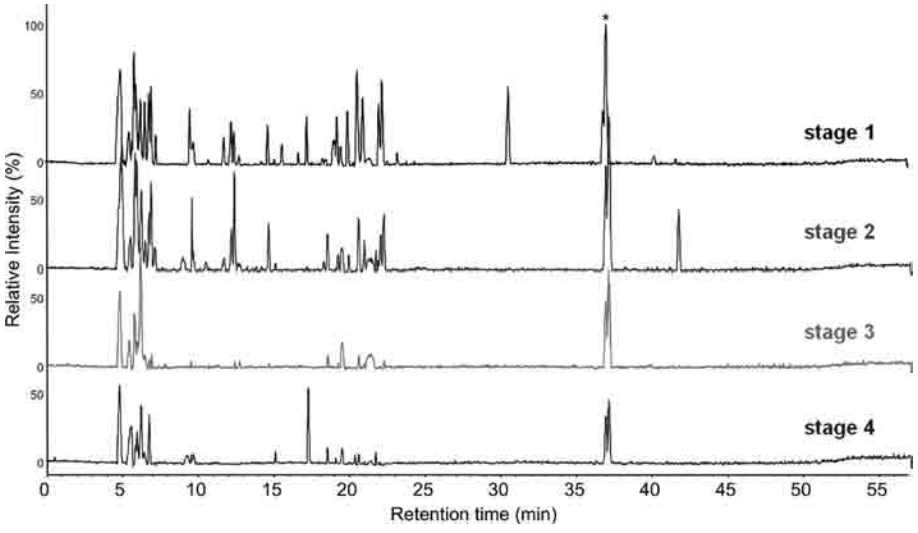

Figure 2. Total ion chromatograms of Jatropha fruits analyzed using LC-Orbitrap-MS (positive ion mode). Chromatograms representative of 3 replicate analyses of fruit from each stage are shown. The scale of intensity of these chromatograms is shown as a percentage scale, in which the highest intensity of the ion shown by asterisk in the chromatogram of the stage 1 was set as 100%.

version of MF Searcher (http://webs2.kazusa.or.jp/mfsearcher/) implemented in the PowerFT, with the maximum number of elements set as 200 for H, 100 for C, 100 for N, 50 for O, 10 for S, and 10 for P. The raw data sets were deposited into the MassBase metabolome database (http://webs2.kazusa.or.jp/massbase/) as accession numbers MDLC1_25527-25534, 25539, 25546-25553, and MDLC1_25559, and are available for free downloading. The annotated data from the raw chromatograms were deposited into the KomicMarket annotated metabolome database (http://webs2.kazusa.or.jp/komics/) as accession numbers KSBA_3, KSBA_4, KSBA_5, and KSBA_6. The alignment matrix with chemical annotation for each ion (see below) is shown in Suppl. Table 1.

Figure 3 shows the distribution of *m*/*z* values for all metabolites detected in the 4 fruiting stages of Jatropha. The total number of metabolites detected in stages 1, 2, 3, and 4 were 5022, 4154, 2438, and 2694, respectively, indicating that the number of metabolites decreases significantly during fruit maturation. In particular, the number of ions with an *m*/*z* value ranging 100–700 decreased significantly between stages 2 and stage 3, suggesting the occurrence of a metabolic transition. The number of metabolites with an *m*/*z* value ranging 100–300 in stage 4 fruit was higher than in stage 3 fruit, although it should be noted that samples of stage 4 fruit did not contain maturing seeds as did the stage 3 samples. Further study is needed to confirm the slight increase of the metabolite numbers by analyzing the stage 4 samples with the seeds. These results suggest that metabolic changes continue even during the later stages of fruit maturation.

To examine the metabolic changes occurring during fruit maturation in more detail, we determined the sequential changes in metabolite quantity using self-organizing mapping (SOM) analysis (Figure 4, Suppl. Table 2), as applied in precedent studies (Hirai et al. 2004). The resulting SOM maps revealed that a dynamic transition occurs between stages 2 and 3. This transition is concomitant with the transition seen in the *m*/*z* distribution (Figure 3), suggesting that significant metabolic changes take place between 3 and 5 weeks after flowering. Some transitions occurring between stages 1 and 2 and between stages 3 and 4 were also observed.

The composition of metabolites detected at each fruiting stage was characterized by annotating the ions and classifying them chemically (Table 1). Using the precise *m*/*z* values of the detected ions as queries, we searched the public metabolite databases KEGG (http://www.genome.jp/kegg/), KNApSAcK (http://kanaya.naist.jp/KNApSAcK/), and LIPIDMAP (http://www.lipidmaps.org/). The number of ions for which the mass matched that of a metabolite listed in one or more of these databases was 1916 (38% of the total

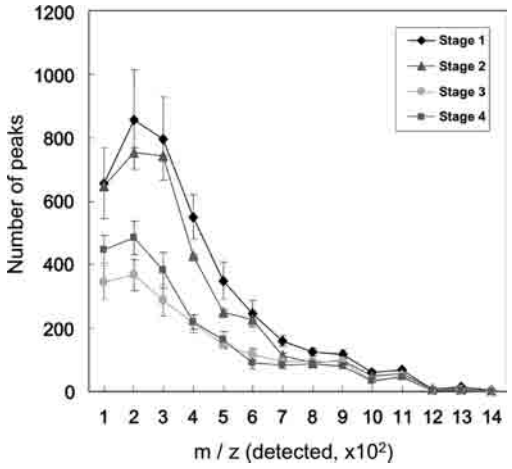

Figure 3. Distribution of *m*/*z* values of the detected metabolites. Three samples of each stage were analyzed by LC-Orbitrap-MS and the resulting data sets were combined into a single set. The bin size was set to 100 to calculate the number of metabolites in each bin. The *m*/*z* values used in this calculation are available at Suppl. Table 1.

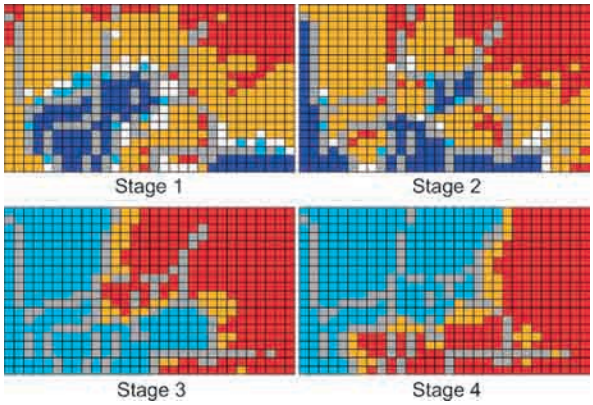

Figure 4. Self-organizing maps of metabolites identified at each stage of Jatropha fruit maturation. The signal intensities of ions obtained from the LC-Orbitrap-MS chromatograms were normalized and averaged between the triplicates for the batch-learning self-organizing map (BL-SOM) analysis (Kanaya et al. 2001). Colors of lattices represent the intensity of ion peaks: red (highest), orange (high), sky blue (low), and blue (lowest). Gray lattices represent blank ones which include no objects (metabolites). See Suppl. Table 2 for the details of the BL-SOM, data processing and the definition of the color chart.

number of ions detected) for stage 1 fruit, 1450 (35%) for stage 2 fruit, 770 (32%) for stage 3 fruit, and 893 for (33%) stage 4 fruit. We analyzed the database hits and distinguished them into two groups; the ions which were annotated as single metabolites or metabolite groups, and the rest of ions which could not be classified into single metabolites or metabolite groups (for the details of annotation procedures, see Suppl. Table 1). The numbers of the single classed ions were 885 (18% in the total number) for stage 1, 660 (16%) for stage 2, 453 (19%) for stage 3 and 488 for stage 4 (18%). These metabolites were classified into 9 chemical categories (Table 1). The number of fatty acid derivative, flavonoid,

Table 1. Number and classification of metabolites identified in samples from each stage of fruit maturation.

Chemical Category	Stage 1	Stage 2	Stage 3	Stage 4	All stages*
Aminocarboxylic acids	115 (92%)	108 (86%)	53 (42%)	66 (53%)	125 (100%)
Sugars	111 (87%)	101 (80%)	71 (56%)	83 (65%)	127 (100%)
Nucleotides	33 (80%)	30 (73%)	27 (66%)	26 (63%)	41 (100%)
Fatty acid derivertives	83 (86%)	75 (78%)	53 (55%)	32 (33%)	96 (100%)
Organic acids	81 (89%)	62 (68%)	48 (53%)	63 (69%)	91 (100%)
Phenolics	224 (75%)	163 (55%)	119 (40%)	143 (48%)	299 (100%)
Flavonoids	168 (89%)	70 (37%)	61 (32%)	49 (26%)	188 (100%)
Steroids	29 (91%)	12 (38%)	5 (16%)	5 (16%)	32 (100%)
Others	41 (67%)	39 (64%)	16 (26%)	21 (34%)	61 (100%)
Total	885 (83%)	660 (62%)	453 (43%)	488 (46%)	1060 (100%)

*The numbers of the single classed metabolites throughout all 4 stages (12 samples in total; see Supplemental Table 1) are shown.

and steroid metabolites decreased substantially with fruit maturation, while there was a tendency toward a decline in the number of metabolites with stage of maturation in the other metabolite categories. Aharoni et al. (2002) reported a metabolic transition from immature to ripe fruit in strawberry, which was detected by nontargeted metabolite analysis using FT/ICR-MS. When the metabolites were extracted with 50% methanol, the total number of metabolites detected with four detection modes (ESI+, ESI−, APCI+ and APCI−) were decreasing during fruit maturation. However, the decrease was moderate when compared with our result of a drastic decrease of total numbers of metabolites during Jatropha maturation. Our observation could be explained by polymerization or incorporation of low molecular metabolites into insoluble materials, which were not detectable by mass spectrometry under the analytical conditions applied in this study. Further investigations are required for a general perspective of the metabolite changing during fruit maturation.

This study demonstrated that dynamic metabolic changes occur during fruit maturation in the perennial deciduous shrub *Jatropha curcas*. However, the detailed annotation of each metabolite present at each stage of fruit maturation remains to be completed. The metabolome data collected here and provided for public use will facilitate the metabolome annotation research.

Acknowledgements

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