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Dissect style response to pollination using metabolite profiling in self-compatible and self-incompatible tomato species

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ABSTRACT

Tomato style is the pathway for pollen germination and pollen tubes growth from the stigma to the ovules where fertilization occurs. It is essential to supplying the nutrients for pollen tube growth and guidance for the pollen tubes. To our knowledge, style also regulates gametophytic self-incompatibility (SI) in tomato species. This study identified the metabolites and monitored the metabolic changes of self-incompatible and self-compatible tomato with self-pollinated or unpollinated styles by gas chromatography–mass spectrometry (GC–MS). A total of 9 classes of compounds were identified in SI and self-compatibility (SC) self-pollinated and unpollinated styles which included amino acids, sugars, fatty acids/lipids, amines, organic acids, alcohols, nitriles, inorganic acids and other compounds. The contents of D-Mannose-6-phosphate, Cellobiose, Myristic acid, 2,4-Diaminobutyric acid, Inositol and Urea were significantly decreased and the rest did not significantly change in SI styles. But change of metabolites content significantly happened in SC styles. In addition, among the total 9 classes of compounds, the different metabolites accounted for a different proportion in amino acids, sugars, amines, organic acids and alcohols compared SC and SI. The result indicated that the physiological changes of styles existed differences in SC and SI after self pollination.

1. Introduction

Self-incompatibility (SI) has attracted a lot of attention in the field of plant molecular biology [1,2]. In flowering plants, SI prevents inbreeding and promotes outcrossing to generate genetic diversity [3]. SI is controlled by both female determinants and male determinants which allow the recognition of non-self pollen [4] and rejection of self pollen [5]. Currently, much work has been done on female determinants-*S*-RNase [6,7] and male determinants-SLF/SFB [8–14]. Tomato reproduction mechanism involves in SI and Self-compatibility (SC) [15]. However, metabolic changes has nor been explored during the pollinations in SI and SC tomato species.

The final recipient of genetic information is regarded as metabolome. Metabolomics can be regarded as complementary to transcriptomics and proteomics [2]. Gene expression, protein stability and metabolic fluxes can be influenced by individual metabolites [16–19]. Plant metabolomics is a new frontier in phytochemical analysis [2]. GC–MS is one of the many kinds of hyphenated mass spectrometry methods for studying plant metabolomics [2]. GC–MS is powerful in identifying metabolites because of its versatility, robustness, technical reproducibility and sensitivity [20]. GC–MS is also an invaluable tool for identifying a larger set of metabolites [21] and understanding the metabolic and physiological processes in tomatoes fruit physiology [22].

We firstly identified metabolites and monitored metabolic changes during the pollinations in SI and SC tomato species by GC–MS. SI is an important genetic mechanism for tomato and its wild relatives. The aim of this study was to characterize biochemical changes of SC and SI tomato styles and provide supplements for SI mechanism of tomato.

Abbreviations: SI, Self-incompatibility; SC, Self-compatibility; GC–MS, gas chromatography–mass spectrometry; BSTFA, *N*,*O*-bis (trimethylsilyl) trifluoroacetamide; TMCS, trimethylchlorosilane; IS, internal standards; UP, unpollination; P, pollination; RTL, retention time lock; TIC, total ion chromatogram; S/N, singal to noise; PCA, principle component; KEGG, kyoto encyclopedia of genes and genomes; NIST, National institute of standards and technology; TCA, tricarboxylic acid.

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2. Materials and methods

2.1. Plant materials

Tomato seeds of SI *Solanum chilense* (LA0130) and SC *Solanum pimpinellifolium* (LA1585) were obtained from the Charles Rick Tomato Genetics Resource Center(University of California Davis http://tgrc.ucdavis.edu/index.aspx). The seeds were germinated in peat pellets and seedlings with three to four leaves were grown on medium containing the perlite: peat (1:1) under a thermoperiod of 26/20 °C (day/night) in greenhouse. Plants were supplied with a commercial fertilizer every week. Tomato flower buds were emasculated one day before anthesis [23] and pollinated with self pollen. Pollinated pistils were collected at 2 h,4 h,6 h,8 h,22 h,24 h, and fixed by 95% ethanol: acetic acid (3:1), cleared by ddH2O and stained with Aniline Blue fluorochrome (Sinopharm Chemical Reagent Co., Ltd., China) in 0.1 M K3PO4 as a stain for callose in pollen tube walls. Slide-mounted pistils were examined using fluorescence microscope (Olympum BX51, Japan) and images were captured at WU330-385 nm and recorded the length of the pollen tube to observe growth behaviors of pollen tubes in SC and SI species in tomato. 24 h unpollination (UP) and self pollination (P) styles were collected from SI *S. chilense* (LA0130) (SIUP/SIP) and SC *S. pimpinellifolium* (LA1585) (SCUP/SCP), respectively, and immediately frozen in liquid nitrogen and stored at −80 °C for metabolite extraction. The three biological replicates of each samples collected for this work were used for metabolite extraction.

2.2. Metabolite extraction and derivatization

Chromatographic grade methanol and chloroform were purchased from Merck Chemicals (Germany). All of the chemicals, such as pyridine, methoxyamine hydrochloride, and *N,O*-bis (trimethylsilyl) trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS), and the reference standards used in the study were purchased from Sigma-Aldrich (St. Louis, MO, USA). L-2-Chlorophenylalanine at 0.3 mg mL^{-1} in water and myristic acid d27 at 0.3 mg mL^{-1} in methanol were prepared and used as internal standards (IS).

An aliquot of 10 mg styles, 100 μL methanol-chloroform (3:1 v/v), and two IS, L-2-chlorophenylalanine and myristic acid d27 (in 5 μL of water and methanol, respectively), were mixed for metabolite extraction at 70 Hz for 300s on a tissuelyser. After centrifugation at 12,000 *g* for 10 min, all the supernatant was dried completely in a vacuum concentrator. An aliquot of 40 μL methoxyamine hydrochloride (15 mg mL^{-1} in pyridine) was added to the residue and incubated at 37 °C for 90 min for methoxyamination. Subsequently, the sample was trimethylsilylated by adding 40 μL BSTFA (with 1% TMCS) and incubated at 70 °C for 60 min. The derivatized samples were cooled to room temperature before being analyzed. For retention time lock (RTL) procedure, a clean RTL sample using the locking compound (myristic acid d27) was additionally prepared.

2.3. Metabolite analysis

Metabolite analysis was carried out by GC–MS method which modified from Roessner et al. [24]. Using an established method contained in the Fiehn metabolomics RTL library (Agilent Inc., CA, USA), five injections of the RTL sample were used to develop a retention time vs. pressure calibration curve. The initial inlet pressure was adjusted to 9.439 psi to achieve the desired retention time of 16.752 min for myristic acid d27. Under the adjusted initial pressure, all samples were analyzed in randomized order by a 7890A gas chromatograph coupled with a 5975C mass spectrometer (Agilent Inc., CA, USA). A DB-5 ms capillary column (30 m × 250 μm inner diameter, 0.25 μm film thickness; J&W Scientific, Folsom, CA, USA) was used to separate compounds. The injector port was heated to 250 °C and injections (1 μL) were performed in splitless mode. Helium (purity > 99.999%) was used as the carrier gas at a constant flow of approximately 1 mL/min. The column temperature was held at 60 °C for 1 min, then increased to 325 °C at 10 °C/min, and held for 10 min. The total run time was 37.5 min. The temperatures of transfer line, ion source and quadruple were maintained at 290, 250 and 150 °C, respectively. Electron impact ionisation mass spectra were recorded with an ionisation energy of 70 eV and EM voltage of 1859 V. Mass spectra were scanned from 50 to 600 amu in total ion chromatogram (TIC) mode after a solvent delay of 5.9 min. Metabolite identification was performed by the Agilent Fiehn metabolomics RTL library coupled with national institute of standards and technology (NIST) mass spectral library (2011) in MSD ChemStation (version E.02.02.1431; Agilent Inc., CA, USA). The RTL procedure assured that the retention time of a metabolite in a sample was the same with that in the Fiehn library. By comparing both mass spectrum and retention time, Fiehn library could greatly enhance the accuracy of metabolite identification, especially for some saccharide isomers whose mass spectra are quite similar.

2.4. Data processing and statistical analysis

The raw data acquired by Agilent GC/MS were imported into ChromaTOF (version 4.50.8.0; Leco Corporation, MI, USA) in NetCDF format. ChromaTOF could automatically compute baseline, find peaks above a signal to noise (S/N) of 100:1, deconvolute (identify overlapped peaks), integrate using specific masses, and align the same compound in different samples. The resulting three-dimensional dataset comprised sample information, peak retention time, and peak intensity. Some artificial peaks generated by noise, column bleed, and by-products in the silylation procedure were removed manually from the dataset. The resulting data were normalized to the area of the IS (L-2-chlorophenylalanine) for further statistical analysis. L-2-chlorophenylalanine was also utilized to assess process variability during sample preparation and data processing. One-way ANOVA (Bonferroni-correction) was performed to identify significant differences among SCUP-SCP (SCUP: SC-Unpollinated; SCP: SC-pollinated) groups and among SIUP-SIP (SIUP: SI −Unpollinated; SIP: SI-pollinated) groups.

2.5. Construction of the metabolic network map, clustering analysis and principle component analysis (PCA)

Identified significant differences metabolites were mapped onto general biochemical pathways according to annotations in kyoto encyclopedia of genes and genomes (KEGG). A tomato style metabolic network map was constructed using Cytoscape 2.8.3 software (http://www.cytoscape.org/). The Mev (MultiExperiment Viewer) 4.8 software was used to perform K-Medians clustering analysis. Principle component analysis was conducted using SIMCA-P 11.5 (http://www.umetrics.com/SIMCA).

3. Results and discussion

3.1. Growth behaviors difference of pollen tube between SI and SC tomatoes

Pistil is the pathway for pollen tubes growth from the stigma to the ovules where fertilization occurs [25]. The pistil is composed of stigma, style and ovary. The stigma provides an environment for pollen grains germination. Style assists pollen tube migration [26]. The extracellular matrix of the style does provide nutritive support for growing pollen tubes. Pollen tube growth in styles is heterotrophic and at the expenses of the stylar reserves. For example,