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Metabolomic analysis of primary metabolites in citrus leaf during defense responses

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Mechanical damage is one of the unavoidable environmental stresses to plant growth and development. Plants induce a variety of reactions which defend against natural enemies and/or heal the wounded sites. Jasmonic acid (JA) and salicylic acid (SA), defense-related plant hormones, are well known to be involved in induction of defense reactions and play important roles as signal molecules. However, defense related metabolites are so numerous and diverse that roles of individual compounds are still to be elucidated. In this report, we carried out a comprehensive analysis of metabolic changes during wound response in citrus plants which are one of the most commercially important fruit tree families. Changes in amino acid, sugar, and organic acid profiles in leaves were surveyed after wounding, JA and SA treatments using gas chromatography–mass spectrometry (GC/MS) in seven citrus species, *Citrus sinensis*, *Citrus limon*, *Citrus paradisi*, *Citrus unshiu*, *Citrus kinokuni*, *Citrus grandis*, and *Citrus hassaku*. GC/MS data were applied to multivariate analyses including hierarchical cluster analysis (HCA), primary component analysis (PCA), and orthogonal partial least squares-discriminant analysis (OPLS-DA) to extract stress-related compounds. HCA showed the amino acid cluster including phenylalanine and tryptophan, suggesting that amino acids in this cluster are concertedly regulated during responses against treatments. OPLS-DA exhibited that tryptophan was accumulated after wounding and JA treatments in all species tested, while serine was down regulated. Our results suggest that tryptophan and serine are common biomarker candidates in citrus plants for wound stress.

[**Key words:** Citrus plants; Primary metabolites; Environmental stress; Metabolomics; Multivariate analysis]

Wound stresses such as mechanical injury and herbivore feeding are unavoidable threats to growth and survival of plants. Damaged tissue allows pathogen invasion and leads to spread of disease into whole plant. Higher plants have evolved defense mechanisms, for instance, wound repair processes are induced in local and systemic area to prevent the penetration of infection (1). Phytoalexins which are an antimicrobial compounds induced by elicitors are accumulated after wounding in higher plants (2).

Some signal transduction pathways are well known to be involved in the *de novo* synthesis of defense related compounds. Jasmonic acid (JA) and salicylic acid (SA) have been reported to play roles as signal molecules in signaling pathways during defense responses (3). It has been reported that mechanical damage induces local and systemic accumulation of JA leading to expression of defense related genes and resulting in defense responses (4). On the other hand, SA has been reported to elicit plant immune systems such as systemic acquired resistance and hypersensitive response (5). However, details in signaling pathways in plant defense mechanisms are still to be elucidated, because of their complexities including changes in metabolites involved in the pathways and interaction of each metabolite. Recently, comprehensive analyses using mass spectrometry (MS) and nuclear magnetic resonance (NMR) have been applied to elucidation of the defense mechanisms against abiotic stresses. Metabolomic approaches have been in the spotlight as a powerful tool to gain comprehensive information of metabolic network and to identify biomarkers related to defense mechanisms (6).

Citrus plants belonging to Rutaceae originated in Asia and now are cultivated all over the world. Citrus fruits are rich sources of bioactive compounds which show pharmacological activities such as antioxidant, antimicrobial, anti-tumor and anti-inflammatory activity (7), and thus citrus family is one of the most commercially important horticultural plants. Despite well-studied pharmacological properties of this family, reports of physiological and biological properties during defense responses against environmental stresses in citrus plants are quite limited. Citrus plants are highly diverse due to their diversity derived from asexual reproduction and sexual compatibilities between *Citrus* and related genera, and thus, it has been difficult to find common physiological behaviors among them. Moreover, since these plants are commonly grown in fields, it is difficult to carry out researches on their physiology under strictly controlled conditions. However, it has been well known that citrus plants are seriously suffered from insect pests accompanied with injury and post-wounded pathogen infections (8). It is necessary to provide insight into their biology and physiology during wound responses for cultivation and protection of this plant species. Our previous report concerning changes in volatile compounds during defense responses against

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wound stresses suggested that hexanal and α-farnesene were induced among tested citrus plants (9). Wound stimuli elicit not only volatiles but whole metabolic network including primary metabolites. In *Citrus unshiu* fruit, high temperature treatment was reported to induce accumulation of 11 amino acids including phenylalanine, tyrosine and tryptophan while low temperature induced ornithine and glutamine (10). However, whether these metabolic changes are common responses among different kinds of stresses and among tissues is still unknown. In this report, we surveyed changes in primary metabolites during responses against wounding, JA and SA treatment using gas chromatography−mass spectrometry (GC/MS) in order to find common defense related metabolites among citrus plants.

MATERIALS AND METHODS

Plant materials The young and intact (non-wounded and non-infected) leaves of seven citrus species, valencia orange (*Citrus sinensis*), villafranca lemon (*Citrus limon*), marsh grapefruit (*Citrus paradisi*), beni unshiu (*Citrus unshiu*), kishu marumikan (*Citrus kinokuni* Hort. ex Tanaka), hirado buntan (*Citrus grandis*), and wako hassaku (*Citrus hassaku* Hort. ex Tanaka) were harvested and collected from mature trees on the Yuasa Experimental Farm, Wakayama Prefecture in July 2014. Ten leaves were collected for each species as 10 biological replicates.

Sample preparation The freshly excised leaves were immediately exposed to mechanical wounding, JA, and SA treatment according to the previous report (9). The mechanically wounded leaves were floated on distilled water to avoid desiccation and incubated at 25°C for 24 h. For JA or SA stress conditions, to minimize mechanical injury, the leaves that were cut at the end of petioles were placed in such a way that the edge of the petiole was in contact with the bottom of a glass bottle, soaked with 0.2 mM JA or SA aqueous solution, and incubated at 25°C for 24 h. For control samples, freshly excised leaves were immediately floated on distilled water and incubated at 25°C for 24 h. The treated leaves were frozen in liquid nitrogen and then, stored at −80°C until freeze dried.

Extraction and derivatization procedure Extraction and derivatization were carried out according to the method of Fukusaki et al. (11). 15 mg-aliquots of freeze dried leaves were homogenized with Lyzing Matrix A (Q-biogene, Inc., CA, USA) using FastPrep Instrument (Q-biogene, Inc.) and then extracted with 1 mL of a mixture of methanol/water/chloroform (2.5:1:1 v/v/v). 60 μL mL of 0.4 mg mL^{-1} ribitol solution was added as an internal standard, followed by extraction using thermomixer at 37°C with the mixing frequency of 1200 rpm for 30 min. The mixture was then centrifuged at 16,000 rpm for 3 min. The supernatant was transferred to a new microcentrifuge tube and mixed with 400 μl of H_2O. The solution was centrifuged and resulting polar phase (upper phase) was transferred to a new microcentrifuge tube. Methanol in the solution was evaporated using centrifugal evaporator, and resulting water layer was then freeze dried. Derivatizations were carried out by adding 100 μl of 20 mg mL^{-1} methoxyamine hydrochloride in pyridine and shaking at 30°C for 90 min, followed by silylation with 50 μl of *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide at 37°C for 30 min.

GC/MS analysis The GC/MS analysis was performed using a GCMS-QP2010 Ultra equipped with an AOC-5000 Plus autosampler (Shimadzu, Kyoto, Japan). 1 μl of derivatized sample was injected into the injection port at 230°C. A CP-SIL 8-CB MS capillary column [30 m × 0.25 mm (0.25 μm), Agilent Technologies, CA, USA] was used, and helium was used as the carrier gas at a linear velocity of 39.0 cm s^{-1}. The oven temperature was set at 80°C for 2 min, and the temperature was then increased to 330°C at a rate of 15°C min^{-1} and maintained for 6 min. The mass spectra were obtained in electron ionization mode at 70 eV with a scanning range of *m/z* 85−500 and a scanning speed of 20 scan s^{-1}. The MS ion source and interface temperatures were 200°C and 250°C, respectively.

Data analysis GC/MS peak detection and alignment were carried out using MetAlign software (www.metAlign.nl). All detected peaks were picked up through this process, and the resulting csv files were applied to Aloutput software (12) for peak identification by retention index and EI spectrum. Calibration of retention time was carried out using *n*-alkane mixture, and peak intensity was standardized by the intensity of internal standard ribitol. The resulting data was processed by PCA and OPLS-DA using SIMCA ver. 13.0.3 (Umetrics, Umeå, Sweden). Pareto scaling method was applied with data set before PCA or OPLS-DA processing. The Aloutput processed data sets were also applied to MeV (Multiple Experiment Viewer) stand-alone software ver. 4.8 (www.tm4.org) for HCA.

RESULTS AND DISCUSSION

Composition of metabolites in seven citrus species Metabolites from leaves of seven citrus species are shown in Table 1. Although some peaks were not identified yet, 28 organic acids, 21 amino acids, 13 sugars and sugar alcohols, and 7 nitrogen containing compounds were annotated by Aloutput software using retention indices and detected masses. To compare differences in metabolic profiles among species, compositions of these compounds in intact leaves were then applied to PCA. Fig. 1 shows results of PCA for intact leaf metabolites in seven cultivars. The clusters of *C. sinensis*, *C. limon*, and *C. unshiu* were located in right-hand region in PCA score plot, indicating that composition of metabolites in these species were different from other species. The loading plot showed that malic acid [**31**] and 4-aminobutyric acid [**36**] contributed to clustering of *C. sinensis* while proline [**20**] and sucrose [**86**] were responsible for clustering of *C. unshiu*. Although there was high quantitative variation among individual samples, *C. limon* formed the separate cluster from other species, and according to loading plot, the clustering was due to the high content of sorbose [**62**] and glucose [**65**]. *C. paradisi*, *C. kinokuni*, *C. grandis*, and *C. hassaku* were located in left-hand region in score plot, however, loading plot showed no specific compound contributing to sample clustering. PCA score plot showed that each citrus species formed independent clusters, indicating that composition of primary metabolites in citrus leaves differs among species. All of citrus plants were derived from citron (*Citrus medica*), pummelo (*C. grandis*), and mandarin (*Citrus reticulata*). It has been suggested that citron-derived *C. limon* is genetically separate species from pummelo-derived (*C. paradisi* and *C. hassaku*) and mandarin-derived species such as *C. sinensis, C. unshiu* and *C. kinokuni* (13). Each cluster in the PCA score plot may reflect the genetic background of each species.

HCA for evaluation of stress responses To explore trends in the metabolic changes in primary metabolites among seven species in citrus leaves in response to wounding, JA and SA treatment, primary metabolite contents were compared using heat map and HCA (Fig. 2). SA treatment of *C. sinensis*, *C. limon*, *C. paradisi*, and *C. unshiu* formed the same cluster while that of *C. kinokuni*, *C. grandis*, and *C. hassaku* was included in independent clusters. In the case of *C. grandis*, all of wounding, JA and SA treatment formed the same cluster, indicating that metabolic profiles in this species reflect genetic background rather than stress responses. HCA for metabolites showed that amino acids, such as aspartic acid, methionine, leucine, isoleucine, tyrosine, valine, lysine, phenylalanine, and tryptophan, included in the same cluster, and thus, these amino acids were suggested to exhibit the same behavior in response to stress treatment. In higher plants, aromatic amino acids such as tryptophan, phenylalanine, and tyrosine are biosynthesized via shikimate pathway from phosphoenol pyruvate, and valine and leucine are biosynthesized from pyruvate which also derives from phosphoenol pyruvate. In contrast, lysine, methionine, and isoleucine are biosynthesized from aspartate which is synthesized via TCA cycle. Our results suggest that these amino acid biosynthetic pathways are concertedly regulated during stress responses. Although the physiological roles of these amino acids are still unknown, some of these amino acids have been suggested to be involved in responses against abiotic stresses. In wheat and *Arabidopsis*, it has been reported that branched-chain amino acids such as valine and leucine were induced against drought stress (14,15) as well as treatment of abscisic acid, a drought stress related hormone (16). Moreover, isoleucine and lysine, aspartate family amino acids, were reported to be related to the osmotic stress (17). It has also been reported that osmotic stress induced threonine deaminase which is involved in the biosynthesis of 2-ketobutyrate, a precursor of isoleucine, and thus, resulted in accumulation of isoleucine (18,19). Stress-induced lysine ketoglutarate reductase and saccharopine dehydrogenase

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