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Metabolic changes in *Citrus* leaf volatiles in response to environmental stress

Tomonori Asai,[1] Tetsuya Matsukawa,[1,2] and Shin'ichiro Kajiyama[1,*]

Graduated School of Biology-Oriented Science and Technology, Kinki University, 930 Nishimitani, Kinokawa City, Wakayama, 649-6493, Japan[1] and Experimental Farm, Kinki University, Yuasa, Yuasa-cho, Arida-gun, Wakayama, 643-0004, Japan[2]

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Citrus plants are well known as a rich source of VOCs, and several have important roles in defense responses. However, how VOCs are regulated in response to environmental stress is not yet well understood. In this study, we investigated dynamic changes of VOCs present in leaves of seven *Citrus* species (*Citrus sinensis*, *C. limon*, *C. paradisi*, *C. unshiu*, *C. kinokuni*, *C. grandis*, and *C. hassaku*) in response to mechanical wounding, jasmonic acid (JA), and salicylic acid (SA) as determined by gas chromatography/mass spectrometric analysis followed by multivariate analysis (principal component analysis, PCA, and orthogonal partial least squares-discriminant analysis, OPLS-DA). PCA and OPLS-DA suggested that changes in VOC profiles against stress stimuli were much diverse among *Citrus* species. OPLS-DA showed that C6 volatiles, such as hexanal and *trans*-2-hexenal, were induced in response to JA and SA stimuli in *C. sinensis* and *C. grandis*, while the other VOCs were decreased under all tested stress conditions. α-Farnesene was induced in all species except *C. hassaku* after wounding or JA treatment. In addition, α-farnesene was also induced in response to SA stimuli in *C. unshiu* and *C. kinokuni*. Therefore these volatiles can be candidates of the common stress biomarkers in *Citrus*. Our results will give a new insight into defense mechanisms in *Citrus* species.

[**Key words:** *Citrus*; Volatiles; Environmental stress; Metabolomics; Multivariate analysis]

Mechanical damage and herbivore feeding behavior are continual threats to the survival of plants. Therefore, the defense responses of plants against wounding stress have been extensively studied. Plants respond to wounding by induction of numerous defense reactions, such as accumulation of phytoalexin or pathogenesis-related (PR) protein, emission of volatile organic compounds (VOCs), and hypersensitive reaction (HR) (1–3). In these defense reactions, emission of VOCs are involved not only in direct defenses, such as toxins and repellents against herbivores and pathogens, but also in indirect defenses that include recruitment of natural enemy insects against herbivores and elicitation of defense responses in intact receiver plants (4,5). Plant VOCs consist of two major classes of compounds, i.e., terpenoids, such as monoterpene and sesquiterpene hydrocarbons and their derivatives, and C6 green leaf volatiles (GLVs). Terpenoids are formed from the biological precursors, isopentenyl pyrophosphate and its isomer dimethylallyl pyrophosphate, and are a structurally diverse group of plant metabolites. Several terpenoids have been suggested to have roles as antimicrobial or antifeedant compounds in direct defense responses (6,7). GLVs, C6 aldehydes, and alcohols and their esters are synthesized from α-linolenic acid via the lipoxygenase (LOX) pathway. The compounds *trans*-2-hexenal and *cis*-3-hexenol are typically released after wounding damage and mediate plant–plant signaling and intra-plant information transfer in indirect defenses (8,9).

Phytohormones, including jasmonic acid (JA) and salicylic acid (SA), are considered to be major second messengers in plant immunity. Wounding induces temporal and organ-specific JA accumulation (10) that mediates activation of defense-related genes, such as the proteinase inhibitor gene (11,12), and results in induction of a defense response against herbivory and pathogen infections. On the other hand, induction of SA accumulation is caused by insect egg deposition or pathogen infection and results in induction of a set of *PR* genes, systemic acquired resistance, and HR (13–15). Therefore, the JA- and SA-signaling pathways have been suggested to regulate different defense responses. The JA pathway is induced in response to necrotrophic pathogens, while SA is primarily activated in response to biotrophic pathogens (16). It has been demonstrated that mechanical wounding induced GLVs, such as *n*-hexanal and *trans*-2-hexenal, in *Arabidopsis* (17), and induced sesquiterpenoids, such as caryophyllene, α-farnesene, and farnesol, in *Hedychium coronarium* (18). In rice, it has been reported that JA treatment induced terpene synthase and two sesquiterpenes: β-elemene and β-bisabolene (19). In contrast, it has been suggested that JA treatment reduced emission of VOCs, while SA and insect-elicitor treatments increased VOCs in cotton plants (20). In addition, SA and yeast extract treatment upregulated biosynthesis of mono- and sesquiterpenoids and expression of biosynthesis enzymes, which accompanied induction of JA and *LOX* gene expression in *Panax ginseng* (21). Thus, interaction of VOCs and JA- and/or SA-signaling varies among different species.

Citrus plants are well known as a rich source of VOCs, and several components in citrus fruits show bioactive functions such as antimicrobial (22), anticancer (23,24), anti-inflammatory (25,26), and antioxidant functions (27,28). However, little is

* Corresponding author. Tel.: +81 736 77 3888; fax: +81 736 77 7011.
E-mail address: kajiyama@waka.kindai.ac.jp (S. Kajiyama).

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known about the physiological roles of VOCs released by citrus leaves. In addition, there have been few reports on interactions between VOCs and wound/JA or SA signaling in citrus plant defense responses. Therefore, it would be useful to identify the dynamic changes in VOCs and the related signaling molecules during stress response to gain insight into the physiology of citrus plants.

Recently, metabolomic analysis has been attracting attention as a new and powerful strategy for elucidation of complexities of plant signaling networks (29). Gas chromatography/mass spectrometry (GC/MS) is a suitable platform for VOCs analysis because it separates majority of the components and makes it easier to identify compounds by matching mass spectral fragmentation patterns to library spectra. In this study, headspace solid-phase microextraction (HS-SPME) with GC/MS was used to investigate the VOC components of citrus leaves and their comprehensive dynamic changes in response to wounding, and JA and SA stimuli were surveyed.

MATERIALS AND METHODS

Plant material The young leaves of seven *Citrus* cultivars, 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 a mature tree on the Yuasa experimental farm, Wakayama Prefecture in July 2014. Five biological replicates were collected for each cultivar.

Sample preparation The freshly excised leaves were immediately exposed to different stress conditions. For the wounding stress condition, the leaves were pierced with fine needles, which created a wound at 6-mm intervals. The mechanically wounded leaves were floated on distilled water and incubated at 25°C for 48 h. For JA or SA stress conditions, 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 JA or SA aqueous solution, and incubated at 25°C for 48 h. Each of the two stress hormones, JA (Tokyo Chemical Industry, Tokyo, Japan) and SA (Nacalai Tesque, Kyoto, Japan), were adjusted to 0.2 mM after dissolution in dimethyl sulfoxide (DMSO). To avoid influences of DMSO, final concentration of DMSO was adjusted to less than 0.1% (v/v). For control samples, freshly excised leaves were immediately floated on distilled water and incubated at 25°C for 48 h.

SPME–GC/MS analysis Portions (10 mg) of leaves were frozen in liquid N_2, ground into a powder, placed into a 20-ml glass vial, and then 1 ml of saturated sodium chloride solution and 10 μl of 100 ppm *n*-octyl acetate (Tokyo Chemical Industry, Tokyo, Japan) in MeOH (using an internal standard) were added before capping the vial. The GC/MS analysis was performed using a GCMS-QP2010 Ultra equipped with an AOC-5000 Plus autosampler (Shimadzu, Kyoto, Japan). After incubating the samples at 30°C for 10 min with continuous agitation (600 rpm), headspace VOCs were extracted using an SPME fiber coated with 50/30 μm of divinylbenzene/carboxen/polydimethylsiloxane at 30°C for 60 s. The SPME fiber was inserted into the injection port for 60 s at 250°C to desorb the VOCs. 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 45.0 cm/s. The oven temperature set at 50°C for 8 min, and temperature was then increased to 180°C at a rate of 4°C min^{-1} and maintained for 5 min. The mass spectra were obtained in electron ionization mode at 70 eV with a scanning range of *m/z* 10–500 and a scanning speed of 20 scan s^{-1}. The MS ion source and interface temperatures were 200°C and 230°C, respectively.

GC/MS data analysis GC/MS chromatograms were analyzed using GCMS solution ver. 4.11 (Shimadzu, Kyoto, Japan). A majority of the peaks in the total ion chromatograms (TIC) were baseline separated, but some of them were partially overlapped with one another. Peak area measurements of the overlapped peaks were individually performed with use of the waveform progress. The mass spectrum data were compared against spectra in the NIST reference library (NIST11, NIST11s) of the GC/MS data system for identification of volatile compounds. Retention indices (RIs) from the literature were used for identification of volatiles. The TIC peak areas of all compounds relative to that of the internal standard were used for multivariate analysis. Principal component analysis (PCA) and orthogonal partial least squares-discriminant analysis (OPLS-DA) were performed using SIMCA ver. 13.0.3 (Umetrics, Umeå, Sweden). Pareto scaling was applied to the data processing before PCA and OPLS-DA.

RESULTS AND DISCUSSION

Composition of volatiles in *Citrus* leaves Volatile components extracted from the leaves of the seven *Citrus* species are shown in Table 1. Identification of the components was based on comparison of their RIs with literature data and comparison of their mass fragmentation patterns to those in the NIST library. Fifty compounds were identified and divided into five groups on the basis of their chemical structures: eight aldehydes, nine alcohols, one ester, 15 monoterpene hydrocarbons, and 17 sesquiterpene hydrocarbons. The ratios of each chemical group on the basis of the total ion current peak area measured by GC/MS are listed in Table 2. Monoterpene hydrocarbons constituted the main part of the leaf VOCs among all of the tested cultivars. The ratio of aldehydes in the *C. limon* leaves was highest among the cultivars tested because of the high percentages of β-citral and α-citral (Table S1). Monoterpene aldehydes were not detected in *C. unshiu*, *C. kinokuni*, *C. grandis*, and *C. hassaku*, but aldehydes derived from fatty acids were included in those cultivars. Although both *C. kinokuni* and *C. grandis* were characterized by high ratios of alcohols ($22.04 \pm 3.86\%$ and $17.71 \pm 1.81\%$, respectively) in the total VOCs, the main component of *C. kinokuni* was linalool, a monoterpene alcohol, while that of *C. grandis* was 1-octanol, an aliphatic alcohol, which suggested that the profile of each chemical group varied among the seven *Citrus* cultivars.

PCA for evaluation of wound, JA, and SA treatment To assess the influences of wounding, JA treatment, and SA treatment on metabolic changes in VOCs in citrus leaves, metabolite profiling by applying PCA to a GC/MS data set was performed. Responses against each stress were clearly different among seven species (Table S2). JA and SA solutions affect levels of the most VOCs but control solution with >0.1% DMSO did not induce significant changes in the levels of VOCs (data not shown). Thus we think that changes in VOC profiles should be due to these hormones. The results of PCA for *C. limon* and *C. kinokuni* are shown in Fig. 1. *C. limon* samples taken from different treatments tended to cluster into independent groups, but the groups were not clearly distinguished in the score plots [$R^2X_{(cum)} = 0.953$, $Q^2_{(cum)} = 0.885$]. Comparison of the corresponding locations in scores and loading plots showed that the VOCs contributing to separation of wound and SA-treatment clusters were β-pinene [**7**] and D-limonene [**14**]. β-Pinene [**7**] and D-limonene [**14**] were the most abundant components in leaves (Table S1); therefore, changes in the amounts of these compounds may have greatly affected the clustering. The PCA score plot from *C. kinokuni* [$R^2X_{(cum)} = 0.957$, $Q^2_{(cum)} = 0.896$] clearly showed separate clusters among different treatment samples, which indicated that metabolism of VOCs in response to different stimuli were independently regulated from one another. Linalool [**22**] contributed to the formation of the SA cluster, while D-limonene [**14**] and γ-terpinene [**18**] contributed to formation of the JA cluster. These compounds were detected as major VOC components in intact leaves of *C. kinokuni* (Table S1), and thus significantly influenced the PCA score plot to separate the JA and SA clusters. On the other hand, in other cultivars, PCA did not show clear separation among treatments (data not shown). In several plant species, it has been reported that mechanical wounding caused activation of the JA-dependent signaling cascade and resulted in induction of the defense systems, including accumulation of functional secondary metabolites (10,11). However, there was no similarity between the wounding and JA-treatment cluster on any PCA. Changes in the volatile profiles in response to wound stimuli could be regulated by a signaling pathway independent of the JA and SA signal cascades.

OPLS-DA for identification of environmental stress markers To identify useful biomarkers, differences in the VOC profiles in treated samples were confirmed by OPLS-DA. Scatter plots (S-plots) corresponding to the OPLS-DA model between the