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Metabolic Changes during Defense Responses against Wound Stresses in Citrus Plants

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<http://dx.doi.org/10.5772/66159>

Abstract

Citrus plants are well known as a rich source of functional chemicals; however, metabolites involved in defense responses against environmental stresses are not yet well understood. Among environmental stresses, mechanical wounding is a continuous threat toward the growth and survival of plants. Recent advances in analytical technology and informatics enable comprehensive analysis of primary and secondary metabolites. In this chapter, metabolic profiling of leaf metabolites in seven *Citrus* species during responses against wound stress as well as defense-related phytohormone treatments was described. Moreover, we discussed current metabolomic techniques, application of these techniques to researches on *Citrus* defense responses and metabolic profiling-oriented identification of novel compounds.

Keywords: defense response, wound stress, metabolic changes, GC/MS, *metabolomics*

1. Introduction

Wound stresses such as mechanical injury and herbivore feeding are unavoidable and continuous 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 against such attack of natural enemies. For instance, plants accumulate wound-healing compounds such as suberin in response to wounding [1] and prepare defense chemicals including repellents and toxins as well as physical defense reaction such as cell wall reinforcement. Moreover, it has been reported that phytoalexins, antimicrobial compounds produced by plants in response to pathogen infections, are accumulated after wounding [2].

Citrus family is one of the most commercially important horticultural plants and cultivated all over the world. Moreover, *Citrus* fruits have been well known as rich sources of bioactive compounds which exhibit pharmacological activities such as antioxidant, antimicrobial, anti-tumor and anti-inflammatory activities [3]. Despite well-studied pharmacological properties of this plant family, knowledge about physiological and biological properties during defense responses against environmental stresses including mechanical injury is quite limited. *Citrus* plants are highly diverse, and their taxonomy and phylogeny are very complex and confusing because of asexual seed reproduction and sexual compatibility between *Citrus* and related genera. Thus, it has been difficult to find common physiological behaviors among them. Since *Citrus* plants are commonly grown in fields, it is difficult to survey their physiology under strictly controlled conditions. However, it was reported that *Citrus* plants are seriously suffered from insect pests accompanied with mechanical injury accompanied with post-wounded pathogen infections [4]. It is necessary to provide insight into their physiology during defense responses against wound stresses for cultivation and protection of *Citrus* plants. In this chapter, we describe metabolomic approaches to elucidating wound responses in *Citrus* plants.

2. Metabolomic approach to investigate responses to wound stress

As described above, when higher plants face to wound stresses, plant metabolism is drastically changed in order to defend themselves against stresses. This metabolic change involves accumulation of defense compounds such as phytoalexins and lignin-like compounds, regulation of signaling pathway, up-regulation of substrate supplies and increase or decrease of many other specific compounds. This reconfiguration of metabolic network is highly complex, and therefore, details in plant defense mechanism are still unelucidated. To understand the mechanism, comprehensive perspective of regulation of metabolic network must be needed. Recently, the “omics” technologies have been developed to characterize and quantify all of the molecules leading to the phenotype of an organism in non-targeted and non-biased manner. Among “omics” technologies, the term “metabolomics” has been used to address the analysis of low-molecular metabolites. Recent advances in technologies of mass spectrometry (MS) and nuclear magnetic resonance (NMR) as well as bioinformatics such as multivariate analyses and chemical libraries enable the application of metabolomics to varieties of organisms. Metabolomics has become in the spotlight as a powerful tool to gain comprehensive and collective information of metabolic network and to find out biomarkers related to defense mechanisms [5].

Since *Citrus* plants are one of the most diverse plant families, metabolite profiles are expected to be diverse among species. *Citrus* plants are commonly grown in open fields, and therefore, variations in levels of metabolites especially those involved in defense mechanisms may be significant due to environmental factors such as temperature, humidity, wind and irradiation of sunlight. Thus, investigation of defense mechanisms of *Citrus* plants needs non-biased and comprehensive analyses and minimization of data variation caused by environmental factors. For this aspect, metabolomics must be a strong tool to understand the *Citrus* defense mechanisms, because metabolomics includes comprehensive instrumental analyses as well as

multivariate analyses which can find specific valuables from numerous and highly diverse valuables.

3. Metabolomic analysis of leaf volatiles during wound responses

3.1. Volatile compounds in plant defense responses

Plants induce various defense reactions including phytoalexin and/or pathogenesis-related (PR) protein, hypersensitive reaction (HR) and emission of volatile organic compounds (VOCs) in response to wounding [6–8]. Among these, emission of VOCs is involved not only in direct defenses, such as toxins and repellents against herbivores, but also in indirect defenses that include recruitment of natural enemies against herbivores and elicitation of defense mechanisms in intact receiver plants [9, 10]. Plant VOCs consist of two major classes of compounds, that is, terpenoids and C6 green leaf volatiles (GLVs). Terpenoids are one of the most structurally diverse groups of plant metabolites and synthesized from two biological precursors, isopentenyl pyrophosphate and dimethylallyl pyrophosphate. It has been demonstrated that several terpenoids play roles as antimicrobial or antifeedant compounds in direct defense responses [11, 12]. GLVs consist of C6 aldehydes, alcohols and their esters and are synthesized from α -linolenic acid through the lipoxygenase pathway. The compounds *trans*-2-hexenal and *cis*-3-hexenol are typically released in response to wounding and have been reported to mediate plant-plant signaling and intra-plant information transfer in indirect defenses [13, 14]

Citrus fruits are well known as a rich source of VOCs, and several components show pharmaceutical functions such as antimicrobial, anticancer, and anti-inflammatory activities. However, little is known about physiological roles of VOCs released by leaves. To understand the roles of VOCs in wound responses, comprehensive analysis of VOCs during wound defense responses would be useful. In this section, metabolic profiling of VOCs during wound responses was highlighted.

3.2. Method of metabolic profiling of VOCs

To compare responses among species, plant materials used for study should be maintained under the same condition to minimize effects of environmental factors. We usually use plants grown in the same field, and at least five leaves per square meter were used for the study. To investigate the responses against stresses, freshly excised leaves were immediately exposed to stresses, and during the treatments, leaves were placed under strictly controlled condition to avoid influence of environmental factors. The treated leaves were immediately frozen in liquid nitrogen and maintained at -80°C before analysis [16].

For analysis of VOCs, gas chromatography/mass spectrometry (GC/MS) is suitable analytical platform because it separates majority of the components and mass spectral fragmentation pattern of each compounds makes it easier to identify compounds. Sample preparation methods for GC/MS include essential oil extraction such as solvent extraction or headspace extraction. Among them, headspace extraction method using microfiber solid phase (headspace solid phase microextraction, HS-SPME) is most sensitive and useful method for com-

prehensive analysis of VOCs. Various SPME fibers are now available for analyses, and among them, divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber has been well used and recommended for VOCs extraction because this fiber consists of triple phases and thus absorbs wide range of volatile compounds [15]. For chromatographic separation of VOCs, nonpolar CP-SIL 8-CB MS capillary column was used, and helium was used as the carrier gas at a liner velocity of 45.0 cm/s. For annotation of VOCs, the mass spectrum data were compared against spectra in the NIST reference library of GC/MS data system, and retention indices (RIs) from the literature were used for identification of VOCs. Identified VOCs in our previous study using seven *Citrus* species, *C. sinensis*, *C. limon*, *C. paradisi*, *C. unshiu*, *C. kinokuni*, *C. grandis* and *C. hassaku* are listed in **Table 1**.

Chemical group	Compounds	Retention indices	Molecular weight	Mass spectrum data <i>m/z</i> (relative intensities)
GLV aldehydes	Hexanal	802	100	41 (100), 44 (97), 56 (89)
	<i>trans</i> -2-Hexenal	846	98	41 (100), 83 (97), 55 (95)
Fatty aldehydes	Octanal	1005	128	43 (100), 56 (77), 44 (77)
	Nonanal	1106	142	57 (100), 41 (66), 56 (66)
	<i>trans</i> -2-Nonenal	1162	140	43 (100), 55 (95), 41 (92)
Fatty alcohol	1-Octanol	1075	130	56 (100), 55 (77), 41 (65)
Monoterpenes	α -Thujene	927	136	93 (100), 91 (47), 92 (40)
	α -Pinene	934	136	93 (100), 92 (40), 91 (33)
	Camphene	950	136	93 (100), 121 (69), 79 (35)
	Sabinene	973	136	93 (100), 91 (33), 77 (28)
	β -Pinene	977	136	93 (100), 41 (43), 69 (41)
	β -Myrcene	991	136	93 (100), 41 (82), 69 (71)
	α -Phellandrene	1005	136	93 (100), 91 (44), 92 (32)
	3-Carene	1008	136	93 (100), 91 (37), 92 (32)
	α -Terpinene	1017	136	121 (100), 93 (98), 136 (61)
	Cymene	1025	134	119 (100), 134 (32), 91 (24)
	D-Limonene	1031	136	68 (100), 93 (100), 67 (66)
	<i>cis</i> - β -Ocimene	1039	136	93 (100), 92 (42), 91 (37)
	<i>trans</i> - β -Ocimene	1050	136	93 (100), 80 (40), 91 (39)
	γ -Terpinene	1061	136	93 (100), 136 (59), 90 (58)
	α -Terpinolen	1085	136	93 (100), 121 (86), 136 (82)
Monoterpene aldehydes	Citronellal	1154	154	41 (100), 69 (95), 95 (54)

Chemical group	Compounds	Retention indices	Molecular weight	Mass spectrum data <i>m/z</i> (relative intensities)
Monoterpene alcohols	β -Citral	1241	152	41 (100), 69 (86), 94 (30)
	α -Citral	1271	152	69 (100), 41 (85), 84 (27)
	Eucalyptol	1033	154	43 (100), 81 (56), 84 (51)
	<i>cis</i> -Sabinene hydrate	1071	154	43 (100), 71 (97), 93 (58)
	Linalool	1101	154	71 (100), 93 (75), 55 (60)
	Terpinen-4-ol	1180	154	71 (100), 111 (50), 93 (47)
	α -Terpineol	1195	154	59 (100), 93 (66), 136 (47)
	Nerol	1223	154	69 (100), 41 (78), 68 (24)
	Geraniol	1253	154	69 (100), 41 (67), 68 (24)
	Thymol	1293	150	135 (100), 150 (35), 91 (15)
Monoterpene ester	Geranyl acetate	1380	196	69 (100), 41 (49), 43 (47)
Sesquiterpenes	α -Cubebene	1347	204	105 (100), 119 (94), 161 (91)
	α -Copaene	1376	204	161 (100), 119 (97), 105 (96)
	β -Elemene	1390	204	93 (100), 81 (91), 68 (67)
	β -Caryophyllene	1421	204	93 (100), 69 (87), 133 (81)
	α -Bergamotene	1435	204	119 (100), 93 (98), 41 (41)
	Aromadendrene	1439	204	161 (100), 93 (91), 91 (89)
	β -Farnesene	1454	204	69 (100), 41 (62), 93 (56)
	Humulene	1457	204	93 (100), 80 (32), 121 (23)
	Alloaromadendrene	1461	204	93 (100), 91 (93), 105 (93)
	γ -Selinene	1475	204	189 (100), 133 (64), 204 (49)
	γ -Muurokene	1482	204	161 (100), 105 (57), 81 (42)
	β -Selinene	1491	204	105 (100), 93 (98), 107 (93)
	α -Selinene	1498	204	189 (100), 93 (86), 107 (74)
	α -Farnesene	1506	204	93 (100), 41 (68), 69 (60)
	β -Bisabolene	1510	204	69 (100), 93 (84), 41 (71)
	δ -Cadinene	1521	204	161 (100), 204 (55), 134 (55)
	β -Sesquiphellandrene	1526	204	69 (100), 41 (52), 93 (49)

Table 1. Volatile organic compounds (VOCs) detected by GC/MS in Asai et al. [16].

Fifty VOCs were identified with our system, and majority of them is terpenoids, that is, monoterpene hydrocarbon and their derivatives and sesquiterpene hydrocarbons. In addition to terpenoids, two GLC aldehydes, three fatty aldehydes and a fatty alcohol were identified. Among VOCs identified, monoterpene hydrocarbons constituted the main part of the leaf VOCs in all species tested according to ratios of each chemical group on the basis of the total ion current peak area measured by GC/MS, but the profiles of VOCs were different among species [16].

3.3. Evaluation of changes in VOCs profiles during wound responses

It has been well known that phytohormones, such as jasmonic acid (JA) and salicylic acid (SA), are involved in the signaling pathway for induction of plant defense mechanisms. Wound stress induces temporal and organ-specific JA accumulation that mediates to activation of defense-related genes and leads to induction of defense responses [17, 18]. In contrast, SA accumulation is caused by insect egg deposition or pathogen infection and results in induction of *PR* genes, systemic acquired resistances and hypersensitive reactions [19, 20]. It has been suggested that JA- and SA-signaling pathways regulate different defense responses and that the JA pathway is involved in responses to necrotrophic pathogens, while SA is primarily activated in response to biotrophic pathogens [21]. However, correlation between VOCs emission and JA- and/or SA-signaling has been reported to vary among plant families [22–24]. To understand wound defense mechanisms in *Citrus* plants, it would be useful to survey the comprehensive dynamic changes in VOC profiles in response to wounding, JA and SA stimuli.

For comparison of VOC profiles among species and treatments, it must be necessary to handle large amount of datasets. To evaluate significant changes in VOC profiles, statistical analysis should be employed. For metabolomics, multivariate analysis is typically employed to process datasets. In our previous report, VOC profiling was performed by application of principal component analysis (PCA) [16]. PCA can provide an overview and clustering of all the applied datasets by projecting each sample.

The results of PCA demonstrated that *C. limon* (common name: lemon) and *C. kinokuni* (kishu) showed separate clusters among different treatment samples, suggesting that metabolism of VOCs in response to wound, JA and SA treatments was independently regulated from one another. In contrast to these two species, *C. sinensis* (sweet orange), *C. paradisi* (grapefruit), *C. unshiu* (unshu), *C. grandis* (pummelo) and *C. hassaku* (hassaku) showed no clear separation among species.

PCA provides an overview of datasets by unbiased and unsupervised manner, and thus, it shows clear clustering only when the variation within each group is sufficiently less than variation between groups. In contrast, since orthogonal partial least square-discriminant analysis (OPLS-DA) is supervised discriminant analysis that relies on the class membership of each treatment, OPLS-DA should be a powerful tool to evaluate treatment-specific changes in metabolites and thus to find biomarkers in defense responses [25]. *Citrus* plants are commonly grown in open fields, and thus, data obtained from them tend to be high variation when open field grown plant materials are used for researches. In order to find wound-related

compounds, data were analyzed by OPLS-DA. According to our previous OPLS-DA results, the patterns of VOC profile changes in the seven *Citrus* species studied could be divided into four different groups [16]. First group included *C. limon* and *C. kinokuni* which showed increase in most VOC components under all treatments. The highlighted markers in this group were D-limonene and β -pinene in *C. limon* and linalool and γ -terpinene in *C. kinokuni*, respectively. Second group consisted of *C. paradisi* and *C. grandis* which showed decrease of most VOC components under all treatments, and among the VOCs, linalool and sabinene were suggested to be markers in *C. paradisi*, while β -pinene was highlighted in *C. grandis*. Third group consisted of only *C. unshiu* and showed a different trend from other species. Most of the VOCs increased after wounding and SA treatment, but decreased after JA treatment. Final group included *C. sinensis* and *C. hassaku*. In this group, several VOCs decreased by wound and JA treatment, while only slight changes in VOCs were detected after SA treatment. Consequently, VOC responses to stresses were suggested to be different among *Citrus* species. However, two GLVs, hexanal and *trans*-2-hexenal, and α -farnesene, were clearly affected in many of tested species, and thus, these compounds can be candidates of the common wound stress biomarkers in *Citrus* plants, although details in their physiological roles were still to be elucidated.

4. Metabolomic analysis of primary metabolites during wound responses

4.1. Changes in primary metabolism during plant defense responses

Wound stress elicits not only secondary metabolites including VOCs but also whole metabolic network including primary metabolites. Activation of glycolytic pathway in response to wounding leads to energy production as well as substrate production for various defense compounds including defense-related proteins [26]. It has been demonstrated that accumulation of free amino acids was induced in response to environmental stresses. Branched-chain amino acids were induced against drought stress, while aspartate family amino acids were related to the osmotic stress [27, 28]. Moreover, amino acid biosynthetic pathways such as tryptophan pathway have been well known to be involved in biosynthesis of defense compounds [29]. For insight into defense mechanisms in *Citrus* plants, understanding of changes in whole metabolic network must be needed. In this section, metabolomics focused on primary metabolites is described.

4.2. Method of metabolomics of primary metabolites

For analysis of primary metabolites, various platforms including capillary-electrophoresis/mass spectrometry, GC/MS and NMR have been developed. Among them, GC/MS has been well used because of its sensitivity and availability for wide range of compounds, although appropriate derivatization should be needed. Extraction and derivatization methods of plant primary metabolites were well developed for validation of food and beverage materials, and data processing software such as peak alignment and peak annotation by mass spectrum is now commercially and non-commercially available. GC/MS-based metabolomics has been frequently and routinely used for many metabolomic studies.

In our previous study, extraction and derivatization were carried out according to the method developed by Fukusaki et al. [30]. In this method, a mixture of methanol/water/chloroform (2.5:1:1 v/v/v) was used as an extraction solvent for a wide range of polar compounds including primary metabolites, and derivatization was carried out by adding methoxyamine hydrochloride, followed by silylation with *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide. GC/MS peak detection and alignment were carried out using MetAlign software (www.metAlign.nl), and ALOutput software [31] was used for peak identification by retention index and mass spectrum. With this procedure, 28 organic acids, 21 amino acids, 13 sugars and sugar alcohols and 7 nitrogen containing compounds were identified [32].

4.3. Profiling of primary metabolites in *Citrus* leaves

Metabolomic analysis of identified primary metabolites in *Citrus* leaves showed that profiles of primary metabolites in leaves of seven *Citrus* species, *C. sinensis*, *C. limon*, *C. paradisi*, *C. unshiu*, *C. kinokuni*, *C. grandis* and *C. hassaku*, were different among species [32]. Among them, *C. limon* formed separate cluster from other species in PCA score plot. It has been suggested that citron-derived *C. limon* is genetically separate species from pummelo- and mandarin-derived species [33]. Although variations in levels of each metabolite were relatively high, this result may reflect the genetic background of each species.

To assess changes in profiles of metabolites, datasets obtained from wound treated leaves and untreated leaves were statistically analyzed. Hierarchical cluster analysis and OPLS-DA showed that levels in amino acids are high sensitivity to stress treatments, indicating that amino acids are involved in *Citrus* defense mechanisms against wounding [32]. Among amino acids, tryptophan and serine were highly sensitive to stress treatments. Tryptophan was up-regulated after wounding and JA treatments, while serine was down-regulated under the same conditions. In tryptophan biosynthetic pathway, tryptophan synthase catalyzed the conversion of serine and indole to tryptophan, and thus, our result indicated the activation of *de novo* synthesis of tryptophan.

5. Identification of novel wound-stress related compounds

5.1. Bottleneck in metabolomics

As described above, metabolomics is a strong tool to find characteristic biomarkers related to genetic and/or environmental factors. However, compound annotation and identification are major bottleneck in metabolomics, especially in mass spectrometry-based metabolomics. Currently, several MS databases have been established, and increasing amount of MS data is now available to facilitate metabolite annotation. It has been estimated that there are hundreds of thousands compounds in plant kingdom, and many of them belong to secondary metabolites. Secondary metabolites are well known as functional metabolites that play important roles in plant physiology and/or ecology, and therefore, many of researches on plant physiology and biology have been focused on the secondary metabolism. Despite of the importance of secondary metabolites, identification of many of them is still to be carried out, and many of

them are not commercially available. MS databases depend on literatures and analyses of authentic standards, and thus, spectra of secondary metabolites are quite limited. For better annotation in plant metabolomics and understanding of plant physiology, isolation and identification of metabolites are essential.

5.2. Defense-related compounds in *Citrus* plants

Citrus plants are rich source of secondary metabolites that exhibit pharmacological activities. Phenylpropanoids, flavonoids, terpenoids and alkaloids are major bioactive compounds in this plant family, and some of them are known to be involved in defense mechanisms against pathogens. For example, polymethoxyflavanones, such as nobiletin and tangeretin, have antifungal effects on *Penicillium digitatum* [34]. Activities of phenylalanine ammonia lyase and chalcone synthase, which are the key enzymes of phenylpropanoid and flavonoid biosynthesis, have been reported to increase after mechanical stress or pathogen infection in a resistant species, *Poncirus* (*Citrus*) *trifoliata*, but not in a susceptible species, *C. sunki*, and thus, these biosynthetic pathways are considered to be involved in defense mechanisms [35]. However, defense-induced compounds derived from flavonoid pathway are still unknown. In *Citrus* plants, coumarins have been identified as phytoalexins from fruits [36], but information of metabolites involved in leaf defense responses is quite limited.

5.3. Isolation and identification of wound-induced compounds in *Citrus hassaku* leaves

Hassaku (*C. hassaku* Hort ex. Tanaka) is one of the most popular *Citrus* species in Japan, and the fruits have been consumed not only as fresh fruit and juice but also as a source of traditional medicine [37]. Varieties of flavonoids, limonoids and coumarins have been isolated from this plant. Despite of increasing studies on pharmacological compounds, reports of defense-related compounds in this plant are quite limited. From these backgrounds, we focused on wound inducible metabolites in hassaku leaves and carried out metabolic fingerprinting-oriented identification of induced compounds [38].

For detection of wound inducible compounds, hassaku leaves were cut into 5 mm square segments by surgical knife for mechanical wounding. This treatment is unusual in fields, but significantly facilitates the wound responses. Wounded and intact leaves were grounded and extracted with methanol that is a useful solvent to extract a wide range of compounds. Leaf extracts were then subjected to high performance liquid chromatography (HPLC) analysis, and chromatograms obtained from wounded and intact leaf extracts were compared. The HPLC fingerprinting showed that two peaks were occurred only in the chromatogram of wounded leaf extract but not in that of intact leaf extract, suggesting that these two compounds were related to the defense response against mechanical wounding.

To isolate wound-induced compounds, crude extract of wounded leaves was subjected to normal phase open column chromatography, followed by reverse phase preparative HPLC. Isolated compounds were applied to MS analysis and NMR for structural characterization. Spectral analyses revealed that one of wound-induced compounds was hesperetin, a major flavanone in *Citrus* plants and another was a novel compound. This novel compound was

characterized as prenylated furofuran lignan (**Figure 1A**) and suggested to be a dimer of citrusnin-A (**Figure 1B**), which was isolated from *C. natsudaidai* [39]. We named this novel compound as “biscitrusnin-A,” and stereochemical analysis revealed that biscitrusnin-A consists of the racemic mixture of two enantiomeric isomers.

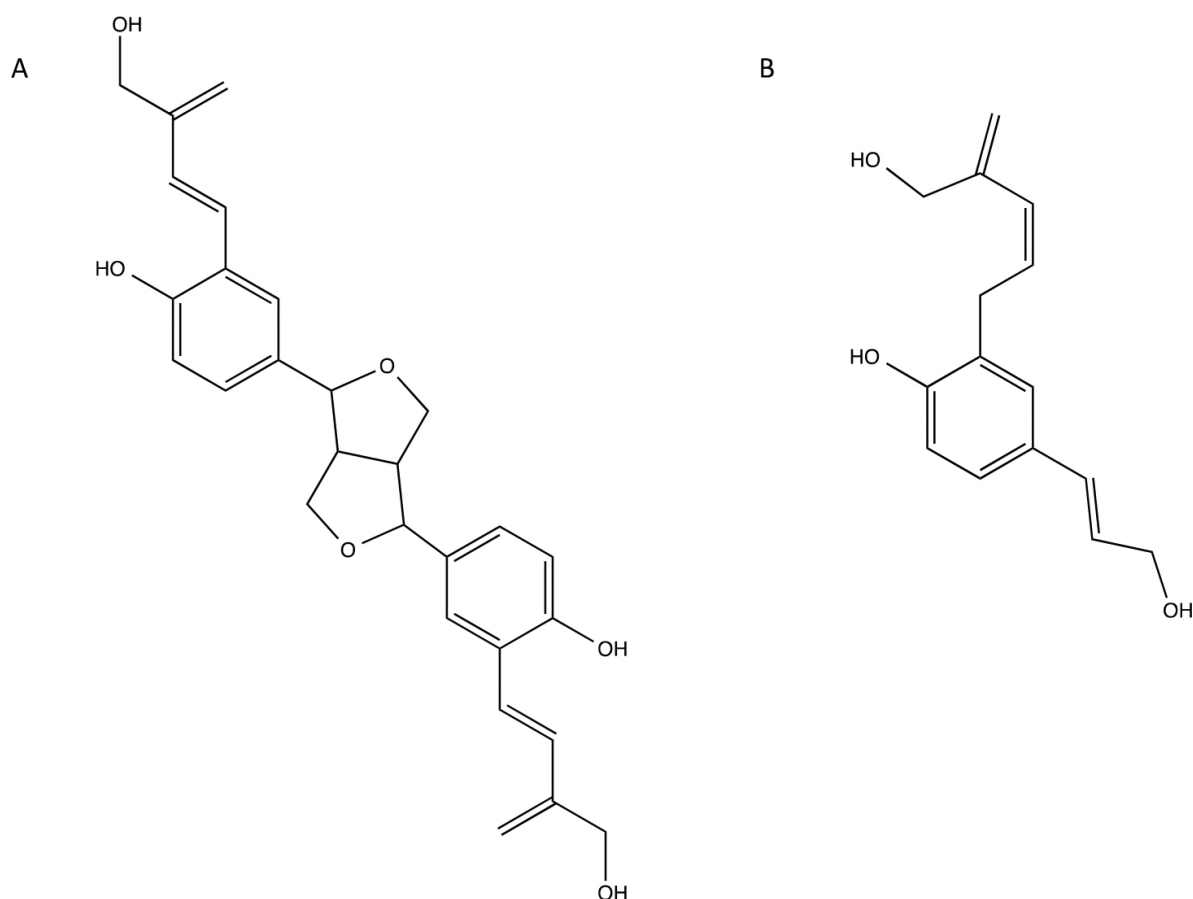


Figure 1. Chemical structures of biscitrusnin-A (A) and citrusnin-A.

To investigate physiological roles of the novel compound, antibacterial activities of these compounds were evaluated against plant pathogens, *Xanthomonas citri*, *X. phaseoli*, *X. oryzae*, *Pseudomonas syringae*, *Clacibacter michiganensis* and *Rhizobium radiobacter* [38]. However, biscitrusnin-A showed no significant activities except for *X. oryzae*. In contrast, citrusnin-A, a monomer of biscitrusnin-A was reported to exhibit high antibacterial activities against almost all of the bacteria tested [38]. It was reported that accumulation of lignans caused by defense responses contributed to cell wall reinforcement rather than direct defense against pathogens [40]. Biscitrusnin-A also might play physical defense roles.

Although prenylated coumarins have been identified from several *Citrus* plants, the report about biscitrusnin-A was a first report about prenylated lignans isolated from *Citrus* plants. Many of prenylated lignans have been isolated from *Zanthoxylum* and *Haplophyllum*, which belong to Rutaceae family as well as *Citrus* [41], and thus, prenylated lignans may distribute in other *Citrus* plants and related genera.

6. Conclusion

In this chapter, metabolic profiling of *Citrus* leaves for investigation of their defense mechanisms is discussed. Metabolomics has been attracting increasing attention as a new and powerful tool for elucidation of complexities of metabolic network. GC/MS-based metabolomics successfully provided the information of metabolites related to wound responses in *Citrus* plants, that is, three VOCs, hexanal, *trans*-2-hexenal and α -farnesene, and two amino acids, tryptophan and serine. In addition, metabolic fingerprinting-oriented isolation resulted in identification of a novel wound-related compound. Although physiological roles of these compounds are still unknown, these compounds can be used as biomarkers in *Citrus* defense responses. Recently, pest management using synthetic pesticides with direct toxicity have been regarded to be undesirable because of their negative effects on ecological environment, and the use of environmentally favorable approaches is required. From this point, plant activators that prime and/or elicit plant defense responses have attracted much attention in crop protection. For development of plant activators, useful biomarkers that indicate defense responses are essential. Various “omics” approaches including metabolomics would be powerful tools to find useful biomarkers of defense responses as well as to elucidate the *Citrus* physiology.

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