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Metabolic movement upon abscisic acid and salicylic acid combined treatments

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Abstract Plant hormones are known to play important roles for maintenance of internal conditions under various environmental stresses. Recent studies revealed that there is a significant cross-talk between abiotic and biotic stress responses. To understand such complex mechanisms, comprehensive analyses at multiple levels are required. In this study, to examine the dynamic interactions between plant hormone responses, we analyzed the metabolic movements of *Arabidopsis thaliana* cultured cells during hormone treatments by NMR metabolic profiling. First, we verified the effect of plant hormone treatments on intracellular metabolites, and detected that the abscisic acid (ABA), salicylic acid (SA), auxin, and brassinosteroid treatment caused metabolic changes. Secondly, since SA and ABA act antagonistically against each other, we monitored dynamic metabolic movements during ABA and SA combined treatments. The response to ABA-only treatment suggested that sugars and amino acids significantly increased. Although SA alone caused fewer metabolic changes, SA caused remarkable metabolic changes when applied in combination with ABA. In addition, our NMR data implied that salicylate glucoside (SAG), which is major metabolite converted from SA, significantly increased in the SA-only treatment but decreased with ABA in a dose dependent manner. These results suggest that ABA and SA cross-talk at the metabolite level in a complicate manner and that the combination of various conditions will provide us with a holistic view of plant stress response mechanisms.

Key words: Cultured cell, metabolome, NMR, plant hormone.

As they are exposed to various environmental stresses, plants have developed elaborate and sophisticated mechanisms to adapt to environmental changes. Understanding of the mechanisms of plant tolerance against environmental stresses such as drought, heat, low temperature, pathogens, or wounds is important to sustain and improve food production. Numerous studies have addressed the physiological status of plants under stress conditions at multiple levels from gene expression to morphological changes (Bartels and Sunkar 2005; Ellis et al. 2002; Feng et al. 2008; Goda et al. 2008; Nishimura et al. 2003; Rajjou et al. 2008; Shinozaki and Yamaguchi-Shinozaki 2007). Recently, metabolomic changes under various stress conditions are gathering attention (Shulaev et al. 2008). Previous metabolomic analyses have clearly shown the metabolic changes in response to environmental stresses, confirming the methodological significance of metabolic analysis for stress response studies. In addition, these metabolic analysis revealed some correlations between metabolic and other "omic" responses to environmental stresses (Baxter et al. 2007; Farag et al. 2008; Osuna et al. 2007; Urano et al. 2009).

In nature, plants are routinely subjected to a combination of different stresses. Thus it is important to describe stress responses under combined stress conditions (Mittler 2006), but there are only a few studies addressing this issue (Broeckling et al. 2005; Rizhsky et al. 2004; Voesenek and Pierik 2008). Plant hormones are known to play important roles in various biotic and abiotic stress responses in plants. Among them, abscisic acid is involved in abiotic stress response while salicylic acid, jasmonic acid, and ethylene are involved in various biotic stress responses. Recent

Abbreviations: ABA, abscisic acid; ACC, 1-aminocycropropane-1-carboxyric acid; BR, brassinosteroid; CK, cytokinin; DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate; GA, gibberellin; HSQC, hetero-nuclear single quantum coherence; MeJA, methyl jasmonic acid; MS, mass spectrometry; NAA; 1-naphthaleneacetic acid; NMR, nuclear magnetic resonance; PCA, principal component analysis; SA, salicylic acid; SAR, systemic acquired resistance

This article can be found at http://www.jspcmb.jp/

studies revealed that there are complex interactions among responses to these plant hormones, indicating that a significant cross-talk between abiotic and biotic stress responses exists (Adie et al. 2007; Fujita et al. 2006; Mauch-Mani and Mauch 2005; Pieterse et al. 2009; Wolters and Jurgens 2009; Yasuda et al. 2008). Because of the complexity of plant responses to combined stimuli and the role of hormones in these responses, we thought it worthwhile to conduct metabolomic analyses of plants as they respond to combined plant hormone stimuli.

Since plants have complex and highly diverse metabolites, it is difficult to analyze plant metabolites comprehensively by a single analytical method, thus mass spectrometry (MS) and nuclear magnetic resonance (NMR) have mainly been used for metabolite detection (Hagel and Facchini 2008; Hall 2006). NMR provides structural information for metabolites without detailed assignments and is a non-selective and property-independent analytical technique, although a drawback is less sensitivity when compared with MS. Thus NMR seems more adapted for "macroscopic" approaches such as metabolic phenotyping. In particular, ^{1}H-NMR is a sensitive and rapid method to detect a large number of metabolites. For example, ^{1}H-NMR metabolic profiling has been shown useful in distinguishing different ecotypes (Ward et al. 2003), transgenic plants or their wild types (Choi et al. 2004; Manetti et al. 2004), and in describing the influence of infection by pathogens on metabolite composition (Abdel-Farid et al. 2009; Choi et al. 2006). Furthermore, there are some reports on the data process of ^{1}H-NMR for metabolic analysis (Hagel and Facchini 2008; Krishnan et al. 2005). Thus, ^{1}H-NMR is very useful at the initial stage in metabolite phenotyping. In addition, the introduction of ^{13}C chemical shifts as a second dimension enables the detection of each metabolite. Although the natural abundance of ^{13}C is low, this problem can be overcome by labeling living materials with ^{13}C labeled compounds (Kikuchi et al. 2004; Tian et al. 2007).

In this study, to examine the dynamic interactions between plant hormone responses, we analyzed the intracellular metabolic movement of *Arabidopsis thaliana* cultured cells using NMR. We found metabolic movements were induced upon hormone treatments and their profiles were different from each other. In addition, we investigated the cross-talk between ABA and SA at the metabolite level. The obtained data suggest that the cellular responses to these hormones are not a simple antagonistic relationship but result from complicated interactions in which metabolic changes different from those in single treatments were induced.

Materials and methods

Plant materials and sample preparation

T87 *A. thaliana* cultured cells were obtained from the RIKEN BioResource Center (Tsukuba, Japan). The cells were incubated and subcultured every seven days in 20 ml of a liquid modified JPL medium (Axelos et al. 1992), in which 0.5% (v/v) glucose was substituted with sucrose, in a 100 ml baffled Erlenmeyer flask at 100 rpm on a rotary shake at 24°C at a 16 h light and 8 h dark cycle. Prior to inoculation, 55 mg cells (wet weight) were filtrated through a 2-mm nylon mesh. ^{13}C-labeled T87 cultured cells were obtained by replacing glucose with [$^{13}C_6$] glucose (>98% ^{13}C, Spectra Stable Isotopes, Columbia, USA) during the incubation for several weeks. For plant hormone treatments, 1 ml of 6-day-old cultured cells were dispensed into a well of 96-deep-well microtiter plates and incubated in the presence of hormones. After the treatment, cells were washed with distilled water, immediately frozen in liquid N_2, and lyophilized. Dried cells (approximately 5 mg/well) were homogenized in 250 μl of potassium phosphate buffer (100 mM, pH 7.0 containing 90% D_2O and 1 mM the TMS group of sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as an internal standard) in a well of 96-deep-well microtiter plate by shaking with a few 2-mm ϕ zirconia beads, and then incubated at 65°C for 15 min. Solutions of three wells were mixed and measured to eliminate the effect of the well position. After centrifugation, the extracted supernatant was transferred into a 5-mm ϕ NMR tube.

NMR experiments

NMR spectra experiments were performed as described previously (Kikuchi and Hirayama 2006; Tian et al. 2007). Briefly ^{1}H-NMR and two-dimensional ^{1}H,^{13}C HSQC spectra were acquired at 298 K on a Bruker AVANCE 700 spectrometer (Bruker, Billerica, MA, USA) equipped with an inverse triple resonance CryoProbe (Brucker) with a Z-axis gradient for 5-mm sample diameters operating at 700.153 MHz for ^{1}H and 176.061 MHz for ^{13}C. Residual water signals in ^{1}H-NMR spectra were suppressed by Watergate pulse sequence (Piotto et al. 1992). For each sample, a total of 16 scans were collected into 65,536 data points over a spectral width of 12,500 Hz. In ^{1}H,^{13}C HSQC spectra, a total of 200 complex f1 (^{13}C) and 2048 complex f2 (^{1}H) points were recorded with 16 scans per f1 increment. The spectral window and offset frequency of f1 and f2 dimensions were 7,042.254 Hz (40 ppm), 11,619.49 Hz (66 ppm), and 9,328.358 Hz (13 ppm), 3,290.88 Hz (4.7 ppm), respectively. The ^{1}H and ^{13}C chemical shifts were referenced to DSS. The spectra were processed using NMRPipe software package (Delaglio et al. 1995). Automatically picked ^{1}H,^{13}C HSQC peaks on the spectra were refined manually and standardized against the calculated noise level of each spectrum. Relative value of each peak was calculated using equation below.

$$\text{Relative values } (I_{rel}) = (I_{sample} - I_{min})/(I_{max} - I_{min})$$

PCA of ^{1}H-NMR spectra

In the PCA, each one-dimensional ^{1}H spectrum between 0.7 and 9.7 ppm was subdivided into 250 segments of sequential 0.036-ppm regions (segments for the signals from DSS and water were removed). Each segment was normalized by the

noise level of the ^{1}H-NMR spectrum. PCA was performed by R software (http://www.R-project.org.).

Real-time RT-PCR

Total RNA was isolated using TRIzol extraction reagent (Invitrogen Japan KK, Tokyo, Japan) from cultured cells. The cDNA was synthesized with 1 μg total RNA using ReverTra Ace Kit (TOYOBO, Osaka, Japan). Real-time PCR was performed in a total volume of 20 μl, containing 10 μl of SYBR Green Realtime PCR Master Mix -Plus- (TaKaRa Bio Inc., Otsu, Japan) and 8 pmol each primer, and 1/40 of the cDNA mixture on LightCycler (Roche Diagnostics, Mannheim, Germany). The amplification program consisted of 50 cycles of 95°C for 10 and 60°C for 1 min. We used the comparative Ct method using *ACTIN2* as a control. The primers At2g43820F, 5′-AGAGTTGGAACTGCATGAGAATGAA-3′; At2g43820R, 5′-CACCGACCCTTGTGGCCTTG-3′; At2g43820F, 5′-TCATCAGCCGGTTCTGTCCCG-3′; At2g43820R, 5′-CCGCAGCTAGACCAAAATCCA-3′; ACTIN2F, 5′-GAGGATGGCATGAGGAAGAGAGAAAC-3′; ACTIN2R, 5′-GCCAGTGGTCGTACAACCGGTATT-3′ were used. Significances of real-time RT-PCR and SAG signal intensity were tested by one-way ANOVA in R software.

Results

Experimental design of this study

To detect the dynamic changes of intracellular low-molecular metabolites responding to exogenous stimuli, we established a method in which multi-samples for ^{1}H-NMR metabolic analyses are easily prepared simultaneously using cultured plant cells. We thought cultured cells are suitable for this study because they are exposed evenly to exogenously applied chemicals and stimulated equally by them. For convenient sample preparation, we used a 96-well-plate format. T87 *Arabidopsis* cells pre-cultured for 6 days were dispensed to a 96-deep well 1 mL each and incubated in a growth chamber. We confirmed that the constant metabolite data was obtained with this incubation method by performing several test experiments (data not shown). Firstly, to monitor metabolic changes caused by exogenously applied plant hormones, we treated cultured cells with various plant hormones [abscisic acid (ABA), 1-naphthaleneacetic acid (NAA) as auxin, brassinosteroid (BR), cytokinin (CK), 1-aminocyclopropane-1-carboxylic acid (ACC), methyl jasmonic acid (MeJA), salicylic acid (SA), gibberellin (GA)] at a final concentration 50 μM for 24 h. Because this study was an untargeted metabolic analysis focused mainly on the water-soluble intracellular metabolites, cells were extracted with 100 mM phosphate buffer and their ^{1}H-NMR spectra were obtained (see materials and methods, Figure 1A, B).

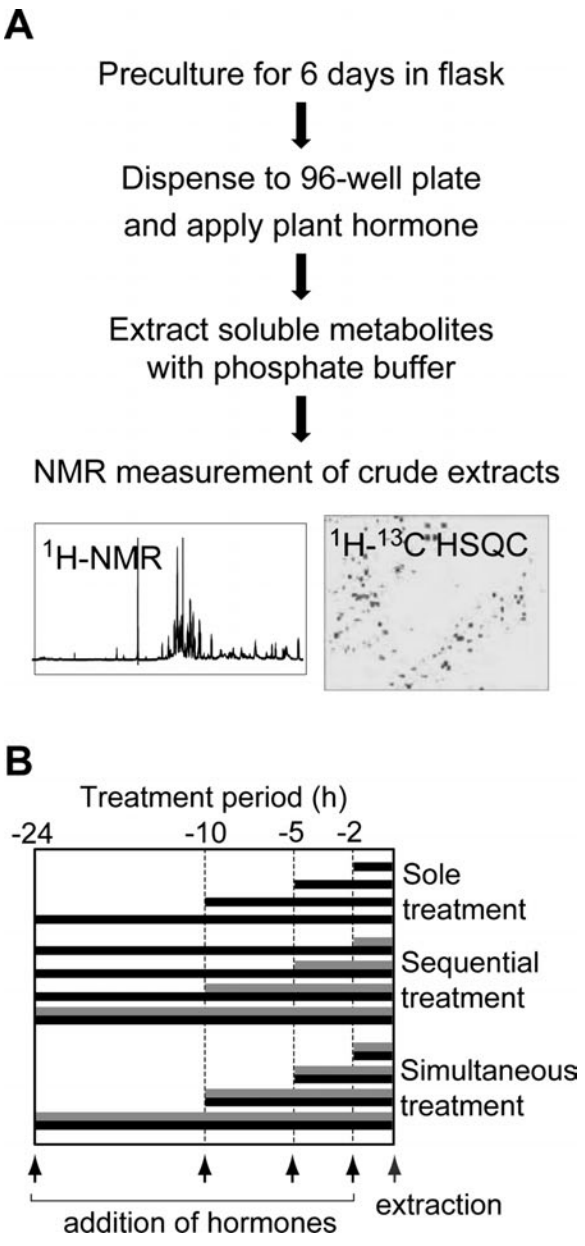

Figure 1. Experimental design of this study. (A) Experimental design of metabolic analysis using NMR. *Arabidopsis* T87 cultured cells were pre-cultured for 6 days and dispensed into wells of 96-well plates for plant hormone treatments. A crude extract was prepared from treated cells and was measured by NMR. (B) Conditions of treatments with plant hormones. Lengths of each bar in the column indicate treatment duration.

Metabolite profiling under hormonal treatments based on ^{1}H-NMR

We found that there were some remarkable differences in ^{1}H-NMR spectra among samples treated with various plant hormones. For example, in the spectrum of ABA or brassinosteroid treated samples, several signals around 3 to 4 ppm that correspond to sugar and amino acid derivatives were stronger than those of the control sample (Figure 2). In the case of brassinosteroid, the signals around 2.5 ppm and 3 to 4 ppm also changed.

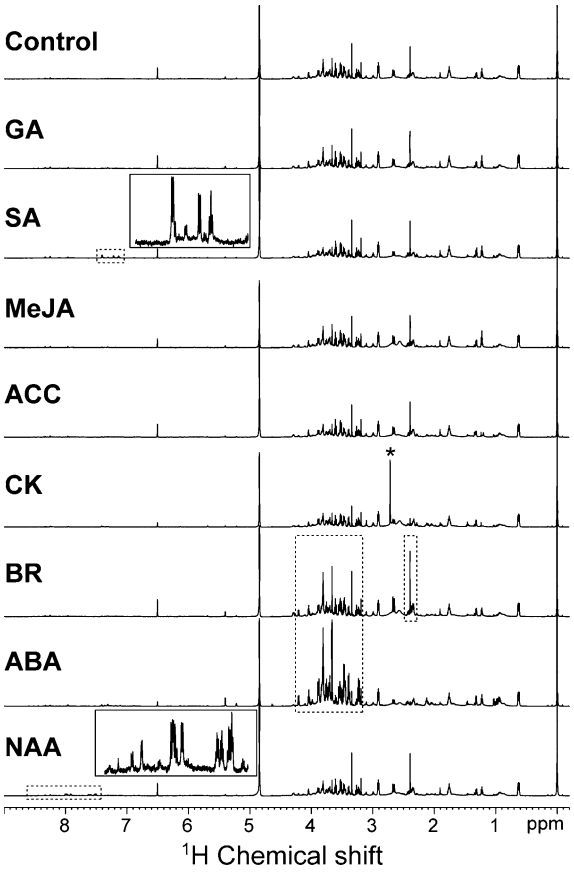

Figure 2. ^{1}H-NMR spectra of crude extracts of cells treated with various plant hormones. The insets in the salicylic acid and auxin samples are the expanded spectra around the aromatic region. The dotted square in brassinosteroid and abscisic acid spectra indicate the signals changed drastically. The signal labeled with an asterisk corresponds DMSO in the plant hormone solution.

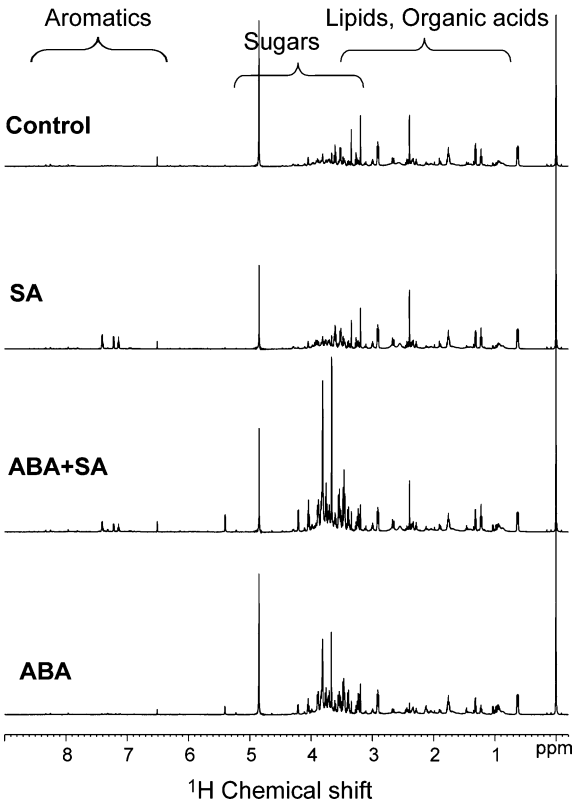

Figure 3. ^{1}H-NMR spectra of crude extracts of the cells treated with ABA and/or SA for 24 h. Brackets above the spectra indicate the regions corresponding to representative compounds.

Interestingly signals for aromatic metabolites were affected slightly by SA or auxin treatment. These data show that ^{1}H-NMR was able to detect the change in the metabolites caused by the hormonal responses (Figure 2).

It has been shown that ABA and SA act antagonistically on each other. However, the molecular mechanism for the cross-talk between ABA and SA remains unsolved. We thought it important to describe this cross-talk at the metabolite level since metabolites are important links between gene functions and phenotypes and reflect the physiological state of the cell. To examine the metabolic changes during ABA-SA cross-talk, we treated cultured cells with several combinations of ABA and SA and measured the metabolites by ^{1}H-NMR. In these experiments, we used ABA at 25 μM and SA at 300 μM, as are used commonly in plant hormone studies (Goda et al. 2008; Hien Dao et al. 2009; Kempa et al. 2008; Krinke et al. 2007; Kuhn et al. 2008). To monitor the time-dependent changes in metabolite profiles, samples were treated with hormones for 2, 5, 10, or 24 h. In addition, cells were treated with ABA and SA simultaneously (ABA+SA) or successively for different periods to test the effect of the order of treatments (Figure 1B). When cells were treated with ABA or SA independently, the ^{1}H-NMR signals corresponding to sugar metabolites or aromatic metabolites increased along with treatment time, respectively. Upon ABA+SA treatment for 24 h, the NMR signals for sugar metabolites were greater compared with the sugar signals for the ABA only treatment (Figure 3).

To gain further insight into the time-dependent metabolic changes caused by ABA and SA treatments, we performed principal component analysis (PCA) on the obtained ^{1}H-NMR spectra (see Materials and Methods). As shown in Figure 4, PCA allowed separating the ABA and SA treatments. The values for principal components 1 (PC1) and 2 (PC2) were clearly different among hormone-treated samples and controls. PC1 separated the samples according to the periods of the ABA and ABA+SA treatments and accounted for 91.9% of the variance within the data. PC2 separated the samples between controls and SA treated samples along with treatment periods and accounted for 4.0% of the variance (Figure 4A). A loading plot showed that the chemical shifts around 3 to 4 ppm corresponding to sugars made a negative contribution to PC1, and those around 3 to 4 ppm and 7 to 7.5 ppm that corresponded to sugars and aromatics, respectively, made

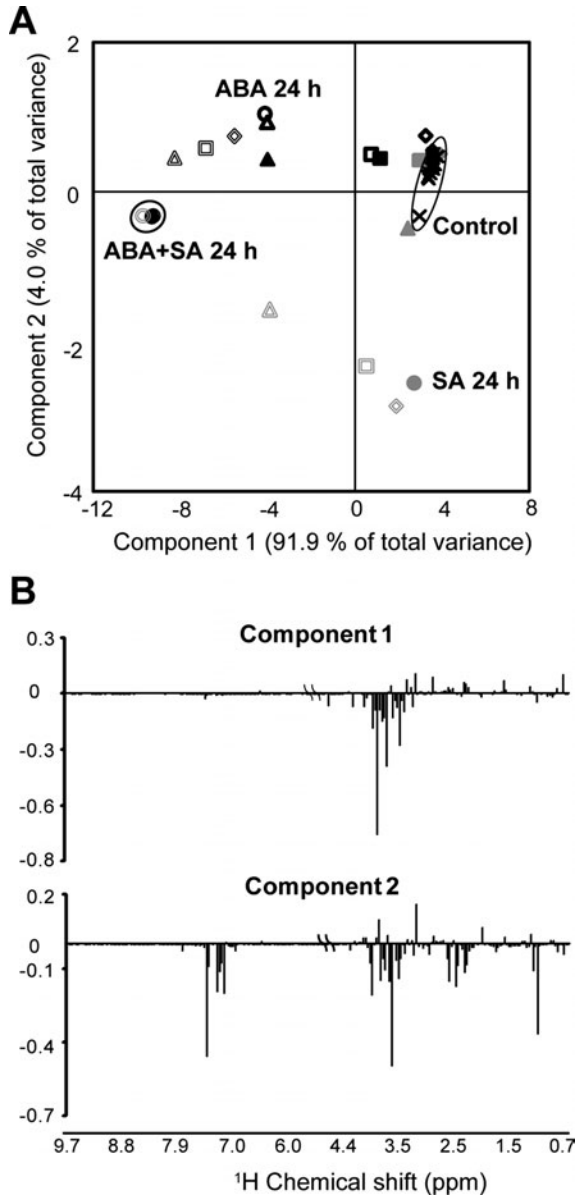

Figure 4. Principal component analysis of ^{1}H-NMR spectra. (A) PCA score plot of ^{1}H-NMR spectra. The signal intensities of each 0.036 ppm segment of NMR spectra except for DSS and water regions were used for the analysis. The intensities of segments were normalized with noise levels. Diamonds, squares, triangles and circles indicate 2, 5, 10, and 24 h treatments, respectively. Open black marks indicate the ABA-only treatments, solid black marks indicate the ABA+SA treatments, solid gray marks indicate the SA-only treatments, black-double-lined marks indicate ABA->SA treatments, and gray-double-lined marks indicate SA->ABA treatments. (B) Loading plot of component 1 and 2 of PCA.

a negative contribution to PC2 (Figure 4B). Interestingly, PCA analysis suggested that the metabolite profile of cells treated with ABA+SA for 24 h was clearly unique from those of the cells treated with ABA or SA alone. Furthermore, these metabolic states occurred independently of the order of hormone treatments.

Metabolic profiling under hormonal treatments based on ^{1}H, ^{13}C HSQC

To obtain more detailed metabolite profiles, we conducted ^{1}H, ^{13}C HSQC measurements. In ^{1}H,^{13}C HSQC, signals originating from ^{13}C atoms bound to ^{1}H in the metabolites are detected. Because there are many peaks with the same ^{1}H chemical shifts, two-dimensional NMR provides better discrimination of the different metabolites. For this experiment, cultured cells were labeled with [$^{13}C_6$] glucose according to our previously described methods (Kikuchi et al. 2004; Tian et al. 2007). To obtain uniformly labeled cultured cells, cells were grown on medium containing [$^{13}C_6$] glucose for several passages. In this study, the labeled glucose in the medium was the sole carbon resource for cultured cells since the growth of the cells are completely dependent on exogenous sugar in our conditions. Thus it is reasonable to think that the signal intensities reflect the amount of metabolites, because the labeling efficiency of each metabolite is presumably constant. In order to detect the effect of hormonal stimuli and their cross-talk, ^{13}C labeled cells were treated with ABA+SA, ABA or SA alone for 2 to 24 h. We detected approximately 600 signals at maximum including ^{13}C-^{13}C coupled signals in ^{1}H,^{13}C HSQC spectra (supplemental data Table S1). Detected peaks were assigned by an in-house Java program (Chikayama and Kikuchi unpublished data), and time-dependent changes in their signal intensities were deduced.

The assigned metabolites and their time-dependent changes based on the obtained ^{1}H,^{13}C HSQC spectra are summarized in Figure 5 and supplemental data Table S2. Forty four metabolites were identified, including 21 amino acids, 9 organic acids, 6 sugars, 3 polyamines, 3 nucleic acid-like compounds, and 7 other compounds. The data showed that the ABA+SA treatment and the sole ABA treatment conferred similar metabolic changes for most detected metabolites, while, in the case of the sole SA treatment, amino acids and other detectable metabolites were almost unchanged during the 24 h treatment, consistent with the data obtained by ^{1}H-NMR. For example, many proteinic amino acids increased along with the treatment period similar to the ABA+SA or the sole ABA treatment. In contrast, beta-alanine, ACC, phenylalanine and unknown P_102 increased extremely under the ABA+SA treatment. In addition, malate, succinate, and fumarate did not change in the ABA+SA treatment similarly in the SA treatment. The 2D-NMR data indicated as with the ^{1}H-NMR data, that ABA and SA do not act antagonistically in a simple manner.

The time-dependent change of putative SAG signal

The aromatic signals which were mainly responsible for

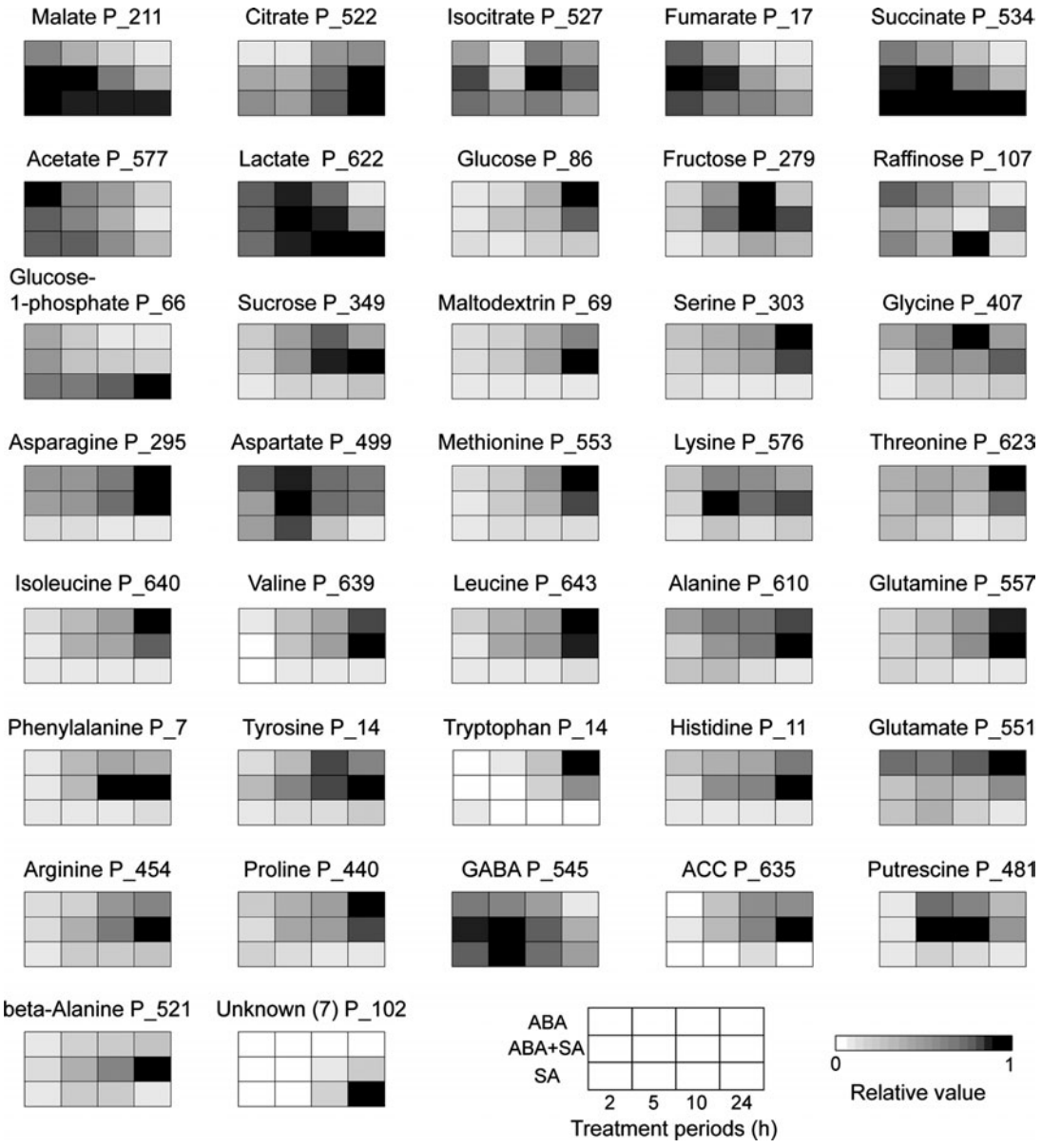

Figure 5. Metabolite movements under hormone treatments analyzed by $^{1}H,^{13}C$ HSQC spectra. The relative values were calculated using relative intensities of metabolites detected in HSQC (see Material and Methods). Only metabolites significantly affected are shown. Upper panel, ABA-only treatment; middle panel, ABA+SA treatment; lower panel, SA-only treatment.

the difference among treatment conditions in ^{1}H-NMR were not detected in $^{1}H,^{13}C$ HSQC spectra. This shows that these signals originated from exogenous SA and not endogenous metabolites, but these signals were not for SA because of their different chemical shifts. Signal intensity of the unknown P_102 in $^{1}H,^{13}C$ HSQC spectra of the ABA+SA treated samples was 4-fold less than that of the sole SA treatment. Furthermore, the chemical shift of unknown P_102 indicated the presence of sugar moiety. It has been shown that exogenous applied SA is converted to glycoside in the cells (Song 2006). Taken together, we putatively assigned this unknown P_102 signal as salicylate glucoside (SAG, salicylate 2-O-beta-D-glucoside). In ^{1}H-NMR spectra, the putative SAG signals decreased in the ABA+SA treatment compared to the SA treatment (Figure 6A). Thus, we investigated the effect of treatments with various concentrations of ABA and SA for 24 h on the SAG levels by monitoring a part of the putative SAG signal in ^{1}H-NMR (Figure 6B). The results suggest a linear negative correlation between ABA concentration and SAG levels regardless of the SA concentration.

It has been shown that SAG is converted from SA by salicylate glucosyl transferase (SAGT), and that the amount of SAG is correlated with the level of transcripts coding SAGT (Song 2006). *Arabidopsis* has two SAGT genes, *UGT74F1* (At2g43840) and *UGT74F2* (At2g43820). We investigated the transcript levels of

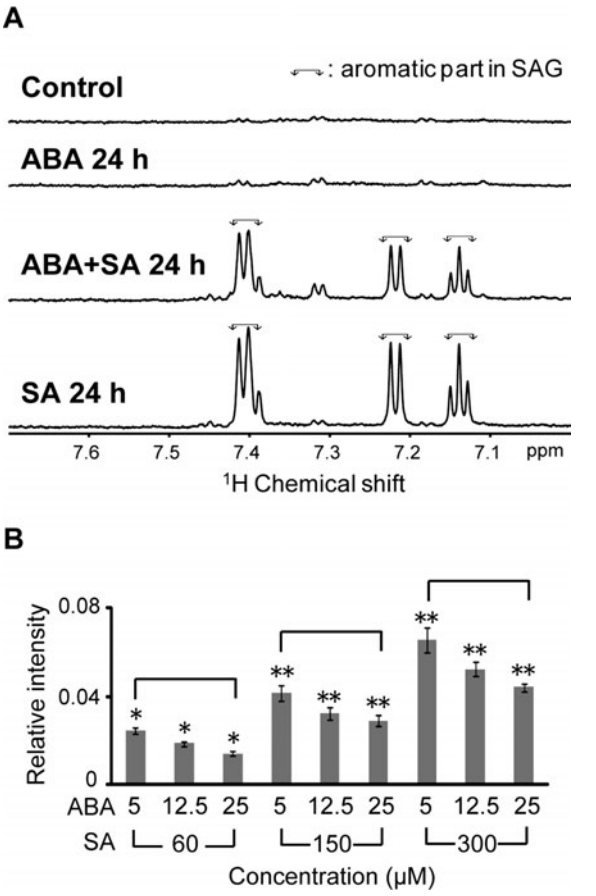

Figure 6. Putative SAG signals in ^{1}H-NMR spectra. (A) Expanded ^{1}H-NMR spectra of the putative SAG signals. The aromatic region of ^{1}H-NMR spectra of the cells treated with SA for 24 h is shown. Arrows indicate putative SAG derived signals. (B) The effects of concentrations of ABA or SA on the SAG levels at 24 h. The signal intensities of a part of SAG signals (7.22 ppm) in ^{1}H-NMR were used. Asterisks,* and**, indicate $P<0.001$ and $P<0.01$ (One-way ANOVA), respectively. Relative intensity was calculated using the DSS signal as a standard.

these two genes by quantitative real-time reverse transcription-PCR (qRT-PCR). However, the PCR products for *UGT74F1* could be hardly detected under any conditions (Supplemental Figure S1). Song reported a similar result that full-length transcript of *UGT74F1* was not detected in *Arabidopsis* leaves (Song 2006). Therefore, we thought that *UGT74F2* is the major contributor for SAG production in this *Arabidopsis* cultured cell, and then we focused on *UGT74F2*. The expression of this gene is known to decrease at 24 h after the infection of pathogens (Song 2006). In the present study, cultured cells were treated for 5 and 10 h with ABA+SA or SA and total RNA was obtained. The data is summarized in Figure 7. The expression level of *UGT74F2* was transiently induced by SA. This SA effect on the expression of this gene was enhanced by ABA.

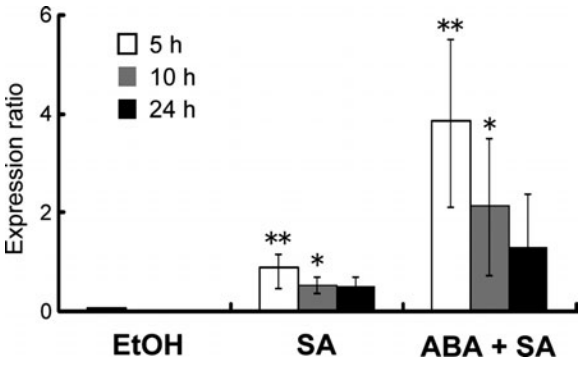

Figure 7. Expression levels of *UGT74F2* in the presence of plant hormones. The expression levels were examined with quantitative real-time RT-PCR (n=3). Asterisks, * and **, indicate $P<0.05$ and $P<0.01$ (One-way ANOVA), respectively. The expression ratio was calculated with cross over points of the *ACT2* gene (efficiency=2.0). White, gray, and black bars indicate 5, 10, and 24 h treatments, respectivel

Discussion

Recently, metabolomic analyses of the response to exogenous stimuli have become a popular way to investigate the physiological effects of environmental stresses. By contrast, there are few studies on the metabolic changes in response to plant hormones that function as signals under various environmental stresses (Hendrawati et al. 2006; Hien Dao et al. 2009; Kempa et al. 2008; Kraemer et al. 2002; Liang et al. 2006). Furthermore, little is known about how combined stresses or plant hormones affect the physiological status of plant cells (Rizhsky et al. 2004). It is essential to clarify the mechanisms that integrate hormonal responses for a comprehensive understanding of the plant stress response.

In this study, we examined the metabolic changes in response to combined treatments with ABA and SA using NMR. Analyzing metabolic composition with ^{1}H-NMR spectroscopy has several advantages. It requires less sample preparation, obtains unbiased information on a wide range of compounds in one experiment, and is rapid and cost effective. We constructed a high throughput method for monitoring metabolic changes using cultured plant cells, which allowed detecting only intracellular metabolic changes affected by plant hormones isocratically. ^{1}H-NMR profiling showed that ABA, SA, brassinosteroid, and auxin treatment generally influenced water-soluble primary metabolites and induced distinct metabolic profiles (Figure 2). These data indicate that each hormone induces distinct metabolic changes, and that monitoring metabolic profiling is useful to describe hormonal responses.

Recent studies indicated that SA and ABA act antagonistically each other (Audenaert et al. 2002; de Torres-Zabala et al. 2007; Mohr and Cahill 2007; Yasuda

et al. 2008). Statistical analysis of ^{1}H-NMR spectra suggest that the ABA+SA treatment induced metabolic changes distinct from those induced by the ABA or SA single treatments. The ABA+SA treatment increased sugars and amino acids more than ABA alone and decreased phenolics less than the SA only treatment. These data indicate that, at least at the levels of primary metabolites, ABA and SA do not have simple antagonistic effects on each other but interact in a more complicated manner. We showed that the order of treatments (SA then ABA or ABA then SA treatments) did not affect the primary metabolite composition in this study, indicating that metabolic status is determined by the physiological conditions at that moment and not by cumulative experience. However, longer-period experiments may be required to provide a clearer conclusion on this issue.

In ^{1}H,^{13}C HSQC spectra analysis, we detected many peaks corresponding to metabolites. In the sole ABA treatment, most of the detected organic acids decreased along with the incubation time. In contrast, sugars, free amino acids and polyamines increased. These observations are largely consistent with the metabolic changes detected under drought stress conditions (Urano et al. 2009), with the exception of raffinose. These metabolites levels were not influenced by the combined SA treatment, suggesting that SA does not perturb ABA action on metabolic processes in osmotic responses. Raffinose is accumulated under drought stress and functions as an osmolyte to maintain cell turgor and to stabilize cell proteins. We could not detect the change of this sugar. This discrepancy might reflect the experimental conditions, specifically the use of cultured cells versus whole adult plants.

It is well known that SA affects secondary metabolites such as phenolic compounds (Kováčik et al. 2009). Several reports have also shown that SA-analogs or elicitor treatments longer than 24 h cause major changes in primary metabolites (Hien Dao et al. 2009; Zulak et al. 2008). Because we investigated mainly primary metabolites in periods shorter than 24 h, we might fail to detect such metabolic changes caused by SA. When applied together with ABA, however, SA seemed to enhance the changes in several metabolites, such as beta-alanine, ACC, and phenylalanine. Beta-alanine has been reported to be increased by abiotic stresses (Fouad and Rathinasabapathi 2006; Hanson et al. 1994; Kaplan et al. 2004; Mayer et al. 1990; Rizhsky et al. 2004). ACC is a precursor of ethylene. ABA triggers ethylene biosynthesis in tomato ripening (Zhang et al. 2009). In addition, ACC production is closely linked to the production of putrescine that is related to polyamine synthesis. Phenylalanine is an important precursor of phenolics. Polyamine and phenolics have some links to reactive oxygen species and senescence, suggesting that the ABA+SA treatments cause such physiological conditions.

In plants, SA exists as a free acid or in forms conjugated by glucosylation, hydroxylation, and methylation, as well as by other hormones. (Lee et al. 1995). Studies of SA metabolism in *Mallotus japonicas*, *Vicia faba*, and *Nicotiana tabacum* showed that SAG is the predominant stable metabolite of SA while SA glucose ester (SGE) is a relatively minor metabolite (Lee and Raskin 1999; Schulz et al. 1993; Tanaka et al. 1990). Conversion of SA into SAG has been reported to be involved in systemic acquired resistance (SAR) in tobacco (Hennig et al. 1993). In *Arabidopsis*, overexpression of SAGT led to increased susceptibility to *Pseudomonas syringae* (Song et al. 2008). Umemura et al. proposed that SAG possibly contributes to SAR by serving as a natural regulator in rice plant (Umemura et al. 2009). We found that the signal intensity of SAG decreased proportionally along with the ABA treatment period and the ABA concentration regardless of the SA concentration. In poplar, SAG is thought to be an acceptor of UDP-glucose involved in sucrose metabolism (Coleman et al. 2007). In our study glucose-1-phoshate, which is a precursor of UDP-glucose, increased in the SA only treatment but decreased in ABA+SA. These results may suggest that ABA affects intracellular SA catabolism by reducing SAG. This idea is consistent with a recent report showing that SAGT activity was decreased by ABA under heat stress acclimation in peas (Liu et al. 2006). Since mRNA levels of *UGT74F2* increased upon treatment with ABA, presumably the transcription of this gene is not the direct target of ABA action.

In this study, we showed that the ABA+SA treatment caused complex situations in metabolic composition which does not indicate a simple antagonistic relationship between the responses to these plant hormones. A combination of various experimental conditions, such as hormone concentration or duration of treatment, will provide us with a holistic view of the mechanism in which plants integrate abiotic and biotic stresses and respond adequately, and will enable us to develop advanced strategies to improve the tolerance of crops to biotic and abiotic stress conditions more efficiently.

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Note added in proof: The patative SAG signals in NMR in this study have been confirmed by comparing with NMR spectrum of a standard compound.