Journal of Chromatography B, 925 (2013) 95–103

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Metabolomic profiling of the response of susceptible and resistant soybean strains to foxglove aphid, *Aulacorthum solani* Kaltenbach

Dan Sato[a,*,1], Hiromichi Akashi[a,b,1], Masahiro Sugimoto[a,c], Masaru Tomita[a], Tomoyoshi Soga[a]

[a] Institute for Advanced Biosciences, Keio University, Tsuruoka, Yamagata 997-0052, Japan
[b] Yamagata Shonai Area General Branch Administration Office, Regional Promotion Division, Tsuruoka, Yamagata 997-1392, Japan
[c] Graduate School of Medicine and Faculty of Medicine, Kyoto University, Yoshida-Konoe-cho, Sakyo-ku, Kyoto 606-8501, Japan

ARTICLE INFO

Article history:
Received 26 October 2012
Accepted 26 February 2013
Available online 5 March 2013

Keywords:
Soybean
Aphid
Pest resistant
Metabolomics
Capillary electrophoresis time-of-flight mass spectrometry

ABSTRACT

Aphid infection reduces soybean (*Glycine max* [L.] Merr.) yield. Consequently, cultivation of aphid-resistant strains is a promising approach to pest control, and understanding the resistance mechanism is of importance. Here, we characterized the resistance of soybeans to foxglove aphid, *Aulacorthum solani* Kaltenbach, at the metabolite level. First, we evaluated aphid mortality and settlement rates on the leaves of two soybean strains, 'Tohoku149' and 'Suzuyutaka', and found that the former had strong resistance soon after introduction of the aphids. The metabolomic response to aphid introduction was analyzed using capillary electrophoresis–time-of-flight mass spectrometry. We found the following three features in the profiles: (1) concentrations of citrate, amino acids, and their intermediates were intrinsically higher for Tohoku149 than Suzuyutaka, (2) concentrations of several metabolites producing secondary metabolites, such as flavonoids and alkaloids, drastically changed 6 h after aphid introduction, and (3) concentrations of TCA cycle metabolites increased in Tohoku149 48 h after aphid introduction. We also profiled free amino acids in aphids reared on both soybean strains and under starvation, and found that the profile of the aphids on Tohoku149 was similar to that of the starved aphids, but different to that of aphids on Suzuyutaka. These tests confirmed that aphids suck phloem sap even from Tohoku149. This study demonstrates the metabolomic profiles of both soybean strains and aphids, which will contribute to the molecular level understanding of mechanisms of soybean resistance to aphids.

1. Introduction

Soybean (*Glycine max* [L.] Merr.) is a major leguminous seed crop that has multiple uses as meal, animal feed, oil, and biofuel. This crop is grown on approximately 6% of the world's arable land, and its production has expanded threefold in the last 20 years [1]. Because of this expansion, quantity control of production is an important issue. Several pathogens, such as bacteria, mold fungi, nematodes, and insects, are responsible for serious yield loss and increasing production costs. The aphid is a known insect pest that causes plant stunting by physical damage, and transmits viruses that lead to non-circulative and circulative yield reduction [2].

Foxglove aphid (*Aulacorthum solani* Kaltenbach) is a polyphagous insect that has sporadically infected various plants in Europe and North America and recently spread worldwide [3,4]. This aphid reportedly transmits soybean dwarf virus, and has caused severe economic loss. In Japan, these aphids have caused early defoliation of soybean by sucking plant sap [5–7], which deforms, spoils, and reduces the size of the soybean grains. These problems have reduced crop yield by 70–90% [6,8]. To date, the following pest control methods have been trialed but found ineffective: insecticides [9], natural enemies [10], transgenic plants for insect resistance [11], and aphid-resistant strains [12].

Cultivation of a resistant strain is a promising method for pest control [13]. Several pest control trials using aphid-resistant strains have been reported in many crops, such as tomato [14], wheat [15], barley [16], melon [17], barrel clover [18], maize [19], sugarcane [20] and soybean [21]. Two soybean strains, 'Dowling' and 'Jackson', have been phenotypically and genetically characterized [22]. Some resistant genes, Rag1, Rag2, Rag3, Rag4, and Raso1, have been identified by linkage mapping [12,23], but the physiological roles in aphid-resistance are unclear.

Recently, the response of soybean to aphids was classified into several stages, including chronic- and acute phases, in studies of the soybean transcriptome. Many hypothetical genes were identified, but the metabolites regulated by these gene products were not elucidated [24]. These data imply that soybean possesses novel

* Corresponding author. Tel.: +81 235 25 0528; fax: +81 235 25 0574.
E-mail addresses: dsparasite@yahoo.co.jp, dsato@z7.keio.jp (D. Sato).
[1] These authors contributed equally to this work.

1570-0232/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jchromb.2013.02.036

metabolomic pathways leading to the defense mechanism. Some metabolic pathways related to phytohormones, such as jasmonic acid, ethylene, salicylic acid, abscisic acid and gibberellic acid, are up-regulated in plant–aphid interactions [25]. However, the mechanism linked to aphid-resistance is poorly understood.

Metabolite profiles can be obtained with metabolomics, which has been applied in soybean to analyze the mechanism underlying salt tolerance [26], flooding stress [27], herbicide induction [28], and the metabolic process in subcellular fractions [29]. Here, we evaluated the resistance of soybeans to aphids using resistant (Tohoku149) and susceptible (Suzuyutaka) strains, and conducted comparative metabolomics analyses with aphid introduction. Our data demonstrated that the concentrations of primary metabolites such as citrate, amino acids, and their intermediates were at higher in Tohoku149 leaves than Suzuyutaka leaves before aphid introduction. These differences were reduced by production of these metabolites in Suzuyutaka leaves 6–24 h after aphid introduction. The concentrations of three metabolites that produce secondary metabolites, such as flavonoids and alkaloids, differed between Tohoku149 and Suzuyutaka, but only at 6 h after aphid introduction. At 48 h, concentrations of TCA cycle metabolites increased in the Tohoku149 leaves. We also compared the free amino acids present in aphids reared on both strains and under starvation, and found that the profile of the aphids on Tohoku149 leaves was similar to that under starvation, and different to that on Suzuyutaka. Metabolite profiling of aphid-resistant and -susceptible strains will provide valuable information for pest control.

2. Materials and methods

2.1. Plant materials and insect culture

Soybean seeds of two strains, Tohoku149 and Suzuyutaka, were gifted by Yamagata prefectural Rice Breeding and Crop Science Experiment Station (Tsuruoka, Japan). Experimental plants were prepared by placing one seed into a plastic pod (⌀9 cm, depth 4.5 cm) filled with sterile culture soil. The plants were gown in a growth chamber at 22 °C with a photoperiod of 16 h light:8 h dark, and used for all experiments after full expansion of the third or fourth trifoliolate. The photosynthetic photon flux density was approximately 100 μmol m^{-2} s^{-1}, as measured with a quantum meter (model QMSW-SS, Apogee instruments Inc., Logan, UT). A foxglove aphid clone was established from a single nymph collected from a tomato seedling in Tsuruoka, Japan in May 2011, and maintained on a continuous supply of Suzuyutaka soybean seedlings under the temperature and photoperiod conditions described above.

2.2. Observation of soybean leaves after aphid introduction

Thirty nymphs (first instar) were gently applied evenly on the adaxial surface of a first trifoliolate leaf of a 'Tohoku149' or 'Suzuyutaka' seedling (five plants each) using a fine tip paintbrush. The target leaf was sealed in the clear vinyl bag (85 mm × 120 mm) with pinholes (approx. 0.9 mm in diameter × 600 pinholes per bag) to keep the aphids on the leaf. The target leaves were photographed with a digital camera at 0, 6, 12, 24, 48, and 96 h after aphid infection. The whole leaf area and area of the lesion caused by aphids sucking sap were calculated based on the digital data.

2.3. Measurement of aphid survival rate

Twenty nymphs (first instar) were gently applied on the target leaf and enclosed in a vinyl bag as described above. The number of survivors was counted each day from day 0 to day 12, and the survival rate was plotted on a Kaplan–Meier plot. This experiment was repeated five times using the first trifoliolate leaves of five plants. The number of survivors in a vinyl bag without a leaf was also counted as a control.

2.4. Measurement of aphid settlement rate

Aphids were applied to soybean leaves as described above with minor modifications. Instead of sealing the target leaf in a vinyl bag, clear plastic tubes (⌀8.5 cm, height 17 cm) were used to cover each seedling pod and the top of the tube was sealed with unwoven fabric. This allowed aphids to move from the target leaf to elsewhere in the pod. The number of aphids on the target leaf was counted at 6, 12, 24, and 48 h after aphid introduction. The aphids in other areas were removed during each count.

2.5. Extraction of metabolites

Thirty nymphs (first instar) were applied evenly on the unifoliolate leaf of each plant described above. After aphid introduction, the aphids were removed and the target leaves were frozen in liquid nitrogen. Intact leaves were used as $t = 0$. Metabolite extraction was performed as described previously [30] with minor modification. The frozen leaves were homogenized using a Shake Master NEO (Bio Medical Science, Tokyo, Japan), and 20–30 mg of homogenates was dissolved in 1000 μL of methanol containing internal standards (20 μmol L^{-1} of methionine sulfone, 20 μmol L^{-1} of 2-(*N*-morpholino)ethanesulfonic acid, and 40 μmol L^{-1} of D-camphor-10-sulfonic acid) to correct migration times in the electropherograms and normalize peak areas to eliminate unexpected systematic bias among runs. After centrifugation at 10,000 × *g* for 10 min, the supernatant (700 μL) was mixed with 280 μL of de-ionized water and 700 μL of chloroform. Insoluble materials were removed by centrifugation at 10,000 × *g* for 10 min, and the remaining aqueous solutions containing hydrophilic metabolites (640 μL each) were filtered using an Amicon Ultrafree-MC ultrafilter (cut off = 3000 Da. Millipore Co., Billerica, MA) with centrifugation at 9100 × *g* at 4 °C for approximately 2 h. The filtrates were dried and stored at −80 °C until use. For preparation of metabolite extracts from the aphids, nymphs (30–40 mg) that were reared on leaves of Tohoku149 or Suzuyutaka, or without leaves, for 12 h were subjected to metabolite extraction as described above. Before analysis, each metabolic extract was dissolved in 25 μL of de-ionized water containing reference compounds (200 μmol L^{-1} each of 3-aminopyrrolidine and trimesic acid) to correct the migration times. To investigate if methionine in the extract was oxidized to methionine sulfone, we also analyzed aphid metabolites with ethionine, a methionine analog, in place of methionine sulfone as the internal standard.

2.6. Instrumentation

The instrumentation and measurement conditions for capillary electrophoresis–time-of-flight mass spectrometry (CE–TOFMS) are described elsewhere [30]. All capillary electrophoresis–electrospray ionization-mass spectrometry (CE–ESI-MS) experiments were performed using an Agilent CE capillary electrophoresis system, an Agilent G6220A LC/MSD TOF system, an Agilent1100 series isocratic HPLC pump, a G1603A Agilent CE–MS adapter kit, and a G1607A Agilent CE–ESI-MS sprayer kit (Agilent Technologies, Santa Clara, CA). The CE–MS adapter kit included a capillary cassette to facilitate thermostating of the capillary. The CE–ESI-MS sprayer kit simplified coupling of the CE system with the MS systems, and was equipped with an electrospray source. For system control and data acquisition, we used G2201AA Agilent ChemStation software for CE and Agilent MassHunter software for TOF-MS.

2.7. Measurement conditions (cationic metabolite analysis)

The CE–TOFMS conditions for cationic metabolite analysis are detailed in an earlier publication [30]. Sample separation was in fused silica capillaries (I.D. 50 μm, length 100 cm) filled with 1 mol L^{-1} of formic acid as the reference electrolyte. Sample solutions were injected at 5 kPa for 3 s, and a voltage of 30 kV was applied. The capillary temperature was maintained at 20 °C, and the temperature of the sample tray was kept below 5 °C. The sheath liquid, composed of methanol/water (50%, v/v) and 0.1 μmol L^{-1} hexakis(2,2-difluoroethoxy)phosphazene (Hexakis), was delivered at 10 μL min^{-1}. ESI-TOF-MS was conducted in positive ion mode. The capillary voltage was set at 4 kV, and the nitrogen gas flow rate (heater temperature = 300 °C) was set at 48.3 kPa. In TOF-MS, the fragmenter, skimmer, and OCT RF voltages were set at 75, 50, and 125 V, respectively. Automatic recalibration of each acquired spectrum was performed using reference masses of reference standards ([^{13}C isotopic ion of protonated methanol dimer (2MeOH + H)]$^+$, *m/z* 66.06371) and ([protonated Hexakis (M+H)]$^+$, *m/z* 622.02896). Mass spectra were acquired at 1.5 cycles/s from *m/z* 50–1000.

2.8. Measurement conditions (anionic metabolite analysis)

The CE–TOFMS conditions for anionic metabolite analysis are described elsewhere [30]. A commercially available COSMO(+) capillary (I.D. 50 μm, length 110 cm) (Nacalai Tesque, Kyoto, Japan), chemically coated with a cationic polymer, was used as the separation capillary. Ammonium acetate solution (pH 8.5, 50 mmol L^{-1}) was used as the electrolyte for CE separation. Before its first use, the new capillary was flushed successively with each of the following solutions for 10 min: the running electrolyte, 50 mmol L^{-1} acetic acid (pH 3.4), and the electrolyte again. Before each injection, the capillary was equilibrated for 2 min by flushing with 50 mmol L^{-1} acetic acid (pH 3.4), and then for 5 min by flushing with the running electrolyte. A sample solution (approx. 30 nL) was injected at 50.8 kPa for 30 s, and a voltage of −30 kV was applied. The capillary temperature was thermostated to 20 °C, and the sample tray was cooled to below 5 °C. An Agilent 1100 series pump equipped with a 1:100 splitter was used to deliver 10 μL min^{-1} of 5 mmol L^{-1} ammonium acetate in 50% (v/v) methanol/water containing 0.1 μmol L^{-1} of Hexakis to the CE interface, where it was used as a sheath liquid around the outside of the CE capillary to provide a stable electrical connection between the tip of the capillary and the grounded electrospray needle. ESI-TOF-MS was conducted in negative ionization mode, with the capillary voltage set at 3500 V. For TOF-MS, the fragmenter, skimmer, and Oct RF voltages were set at 100, 50, and 200 V, respectively. The flow rate of drying nitrogen gas (heater temperature = 300 °C) was maintained at 10 L min^{-1}. Automatic recalibration of each acquired spectrum was performed using reference masses of reference standards ([^{13}C isotopic ion of deprotonated acetic acid dimer (2CH3COOH−H)]$^-$, *m/z* 120.03841), and ([Hexakis + deprotonated acetic acid (CH3COOH−H)]$^-$, *m/z* 680.03554). Exact mass data were acquired at 1.5 spectra/s from *m/z* 50–1000.

2.9. Metabolite identification, quantification, and data analysis

Metabolites were identified by comparison with the accurate *m/z* values and migration times of commercially available compounds. The concentrations were calculated from the peak areas of the compounds, which were normalized by internal standards. Digitalization of the leaf area and calculation of the lesion area were performed using Image J ver.1.45 (http://rsbweb.nih.gov/ij/). The Kaplan–Meier plot was generated using GraphPad Prism ver.5 (Graphpad Software, San Diego, CA). Raw metabolite data were analyzed with our proprietary software, MasterHands [30]. Principal component analysis (PCA) was conducted using JMP (9.0.2, SAS Institute, Cary, NC). Dataset groups were compared using student *t*-tests (two-tailed).

3. Results and discussion

3.1. Validation of CE–TOFMS measurement

In this study, we performed metabolomic profiling in both soybean leaves and aphids. An established protocol was used for measurement of soybean leaf metabolites by CE–TOFMS [31]. However, to our knowledge, this is the first report of CE–TOFMS for aphid metabolite profiling. To validate the measurement protocol we evaluated the linearity between the metabolite concentrations and dilution rates. Good linearity was observed for all amino acids (Fig. S1 and Table S1), with R^2 values between 0.999 (Trp) and 1.00 (Met). The relative standard deviations for six replicate measurements of 19 amino acids (Cys was not detected) were between 1.04 and 3.75% (dilution ratio = 1) and 0.89 and 5.19% (dilution ratio = 10). In the 1/100 diluted metabolite extract, the RSD was between 1.66 and 16.7% except for Met and Phe, which showed small mean values (Fig. S2). These results indicate the measurement protocol is valid for quantification of the metabolites.

Methionine sulfone, an oxidized form of methionine, was used as an internal standard for cationic metabolite analysis. To investigate if methionine sulfone was generated by oxidization of endogenous methionine, methionine and methionine sulfone in the metabolite extract were measured using ethionine as the internal standard. Ethionine is a structural analog of methionine. The endogenous methionine sulfone was not detected (Table S2), which suggests that it is suitable to use it as an internal standard.

3.2. Phenotypic observation of soybean leaves after aphid introduction

The leaves of Suzuyutaka (aphid-susceptible strain) had a phenotype that was distinct from that of Tohoku149 (aphid-resistant strain) after aphid introduction. Aphid feeding produced yellowish-green spots on Suzuyutaka leaves 24 h after aphid introduction, and these spots became clear yellow after 48 h, and then blotches after 96 h (Fig. 1a, lower panel). By contrast, the leaves of Tohoku149 remained almost asymptomatic, with a small number of yellow to brown spots observed 96 h after aphid introduction (Fig. 1a, upper panel). The densitometric data of the target leaves confirmed that the percentage of the affected area of Suzuyutaka was larger than that of Tohoku149 from 12 to 96 h after aphid introduction. For Suzuyutaka, the affected area largely increased in size at 48 h and 96 h after aphid introduction, which was shown by the densitometric data (48 h = 4.9 ± 0.6% and 96 h = 28.8 ± 5.1%, mean ± standard error [S.E.] from triplicate measurements). The aphid, *Aphis glycines* Matsumura, causes serious damage, such as discoloration, leaf distortion, stunting, and desiccation, to leaves from susceptible strains and eventually leads to plant death. By contrast, resistant strains, such as Dowling, show less damage [21]. Unlike the Dowling strain, leaf distortion and growth defects were not observed in Tohoku149 leaves, possibly because of differences in the soybean strain and/or aphid species.

3.3. Quantitative evaluation of aphid resistance based on survival and settlement rates

The resistance to aphids was evaluated quantitatively using the survival and settlement rates. The survival rate was calculated using the number of living aphids on the target leaves. The settlement rate was calculated using the number of living aphids present in the plant culture container but not restricted to the target leaves.

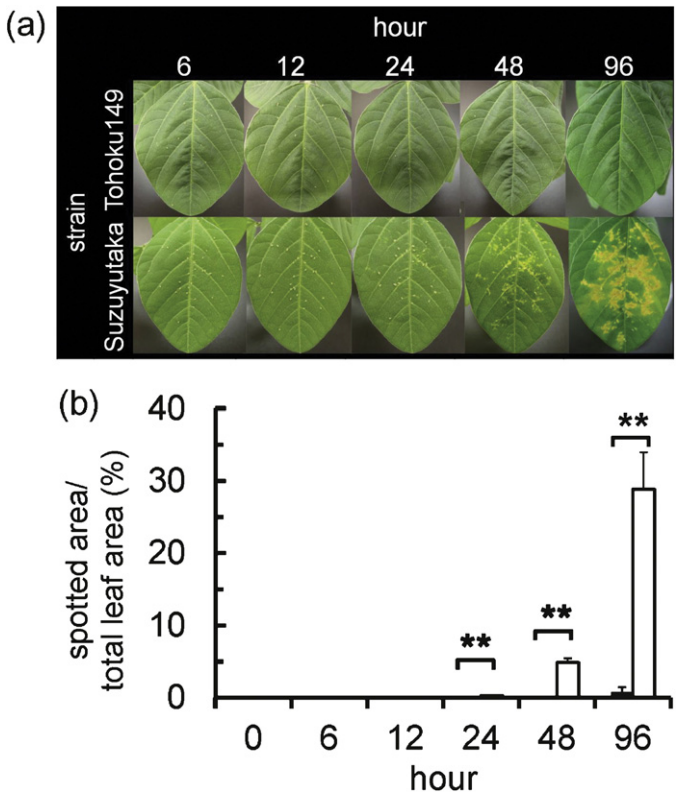

Fig. 1. Changes in the color of the soybean leaves after aphid introduction. (a) Representative pictures of Tohoku149 (upper panels) and Suzuyutaka (lower panels) leaves after 6, 12, 24, 28, and 96 h. Tohoku149 leaves did not show chlorosis when compared to the Suzuyutaka leaves. (b) The relative lesion area was calculated by densitometry (mean ± S.E., $n = 5$). Tohoku149 and Suzuyutaka are indicated by black and white bars, respectively. Asterisks (*) denote statistical significance with $P < 0.01$.

3.3.1. Survival rate analysis

The median survival time, which is the time on the Kaplan–Meier plot that indicates 50% survival rate, of the aphids on Tohoku149 leaves was 4 days, while most aphids were dead 10 days after introduction (Fig. 2a, solid line). By contrast, over 95% of the aphids on the Suzuyutaka leaves were alive at day 10 (Fig. 2a, dashed line), which confirms there is significantly higher mortality on Tohoku149 leaves than Suzuyutaka ($P < 0.01$). The aphids on the Tohoku149 leaves survived more than the aphids under starvation, where all aphids were dead by day 4 (Fig. 2a, dotted line). This suggests that the aphids suck phloem sap from Tohoku149 leaves, and feeding inhibition may not cause the resistance.

3.3.2. Settlement rate analysis

The settlement rate on Tohoku149 leaves was significantly lower ($P < 0.01$) than that on Suzuyutaka leaves 6 h after aphid introduction (Fig. 2b, solid line), and decreased to 0% at 48 and 96 h. This indicates that the aphids moved from the target leaves to other areas of the plant cultivation container or died. This data confirmed the strong resistance of Tohoku149 to aphid infection.

Several soybean strains resistant to aphids have been characterized in field-based studies [32,33]. Although these studies are closely linked to real agricultural production compared to laboratory-based studies like ours, it is not possible to establish the etiology or trace it to a single pathogen. We unambiguously demonstrated the aphid-specific resistance of Tohoku149, while excluding other pathogenic factors. It has been reported that soybean strains such as Dowling, Jackson, PI 200538, PI 243540, PI 567541B and PI 567543C are resistant to *A. glycines* [34–38]. Resistant genes, Rag1 to Rag4, have been identified in some of these strains, and these can be used as genetic markers to screen for resistant strains, but the mechanism of resistance is still unknown.

The settlement rates indicated the number of aphids was reduced on resistant leaves soon after their introduction (Fig. 2b), with half of the aphids moving elsewhere in the plant cultivation container. By contrast, the proportion of aphids still on the aphid-susceptible leaves 6 h after aphid introduction was significantly higher (86%, $P < 0.01$). After 48 h, most of the aphids had moved from the leaves. This result might be related to differences in the aphid feeding behavior on resistant and susceptible strains, which were detected using an electric-monitoring system [39,40]. We speculate that aphid-resistance of Tohoku149 is involved in inhibition of nutrient uptake from the phloem, and an inhibitory factor is continually present in the phloem sap or aphid sucking induces its production.

3.4. Metabolomics analysis of soybean leaves

3.4.1. Overview of the metabolomics analyses

We identified 196 ionic metabolites by CE–TOFMS. The representative extracted ion electropherograms are shown in Fig. 3. First, PCA analysis was performed to evaluate the overall differences in the metabolic profiles of the leaves of Tohoku149 and Suzuyutaka ($n = 5$ for each group). Score plots (Fig. 4) revealed a clear difference in the first principal component (PC1), while almost no difference was observed in the second PC (PC2) for the plots before aphid introduction (0 h). The difference in PC1 dramatically decreased, while the difference in PC2 increased, after aphid introduction (6 h). The trend for changes in the third PC (PC3) was similar to that for PC1 (Fig. S3). The plots of PC1 and PC2 at 0 h and 6 h were clearly different from those 12 h after aphid introduction. These large distances

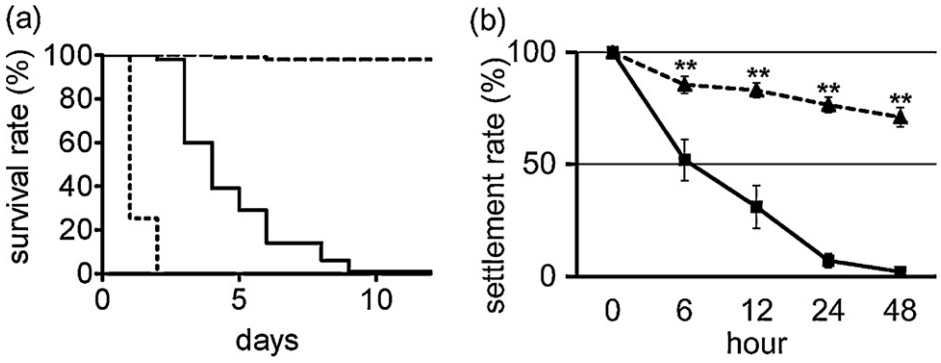

Fig. 2. Evaluation of aphid resistances of Tohoku 149 and Suzuyutaka strains. (a) Aphid survival rate on Tohoku149 leaves (solid line), Suzuyutaka leaves (dashed line), and under starvation (dotted line) as Kaplan–Meir plots. (b) Aphid settlement rates on Tohoku149 (solid triangles) and Suzuyutaka (solid squares) leaves. Mean ± S.E. ($n = 5$). Asterisk (*) denotes statistical significance with $P < 0.01$.

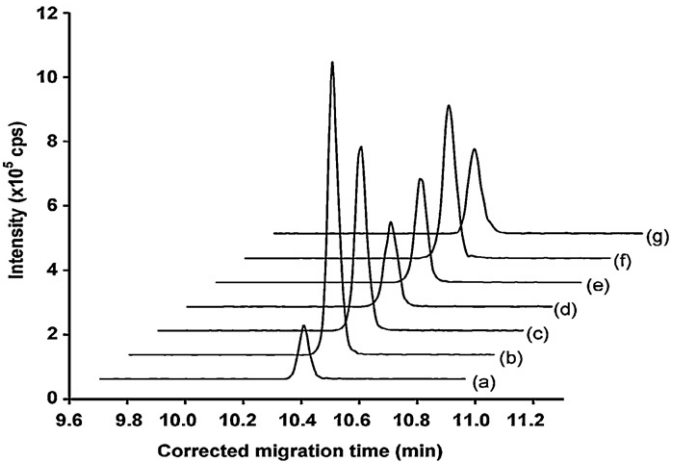

Fig. 3. Extracted ion electropherograms of Lys from aphid-susceptible soybean (Suzuyutaka) leaves as a representative metabolite. Lines (a)–(f) are the electropherograms of Lys from the leaves at 0, 6, 12, 24, 48, and 96 h after aphid infection. Line (g) is for the Lys standard (20 μmol L^{-1}). The *x*- and *y*-axes show the corrected migration time and intensity of the ions (counts per seconds), respectively.

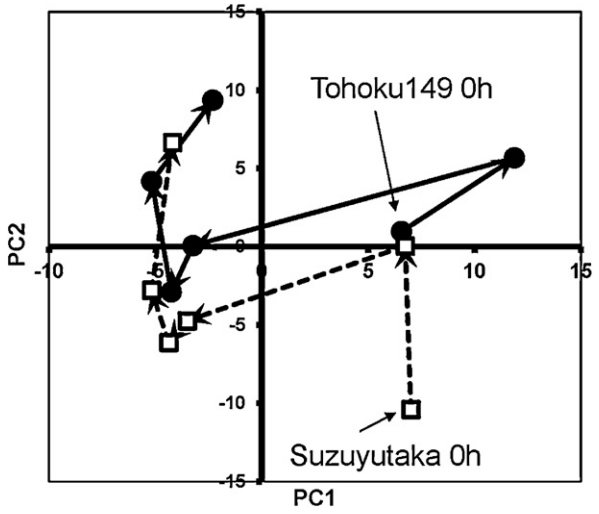

Fig. 4. PCA score plots. The filled circles and open squares show data from Tohoku 149 and Suzuyutaka strains, respectively. The data collected at 0, 6, 12, 24, 48, and 96 h are connected in chronological order using arrows. PC1 and PC2 are the first and second principle components, respectively. The accumulated contribution ratios were 19.2% and 36.2% for the first and second PCs, respectively. The score plots, including PC3 and the loading plots, are shown in the Supplementary Information (Figs. S2 and S3).

between the score plots at 0 h indicate the metabolomic profiles of the two strains are inherently different, and changes in the early stages (0–6 h) reflect the acute response in metabolite levels to aphid introduction. Loading plots (Fig. S4) showed that while metabolites in various pathways had large PC values, the amino acids were dominant (e.g. Asp, Thr, Trp, His, and Med with large PC1 values; and Phe, Tyr, Pro, Ile, Trp, Leu and Ala with large PC2 values). Interestingly, Trp had large PC1 and PC2 values, and underwent a drastic change at the acute response.

3.4.2. Comparison of metabolites between resistant and susceptible strains

The metabolic profiles of components of the TCA cycle, amino acids, and the other metabolites described in this text are illustrated in Fig. 5. Overall, none of the metabolites showed distinct patterns in Tohoku149 or Suzuyutaka throughout the experiment, and several different profiles were observed over the time-course of the experiment for Tohoku149 and Suzuyutaka. For example, amino acids in the downstream pathway of pyruvate and oxaloacetate showed large differences only at 0 h, and metabolites of glycogenesis, such as 3-phosphoglycerate and phosphoenolpyruvate showed relatively large differences at 48 h. Through the whole observation period, comparison of metabolite concentrations in the resistant and susceptible soybeans leaves showed 55 significant differences (Table 1). Before aphid introduction (at 0 h), 25 metabolites, which account for almost half of the total difference, were detected at different concentrations in these strains. The most outstanding feature was that the citrate concentration in Tohoku149 was 34-times higher than that in Suzuyutaka at 0 h. This rapidly reduced along with the time after aphid introduction as follows: 34-times at 0 h, 2.3-times at 6 h, and 0.9-times at 12 h. This occurred because of the increasing citrate concentration in Suzuyutaka leaves (data at 6 h and 12 h are not shown, Fig. 5). Up-regulation of the citrate synthase gene by aphid-feeding has been reported in sorghum [41]. It is conceivable that the high citrate concentration observed in the present study is involved in protection of the leaves from aphids. By this reasoning, the initial high citrate concentration of Tohoku149 contributes to its resistance, and Suzuyutaka accumulates citrate after aphid introduction to acquire resistance.

Among the 25 metabolites that showed higher concentrations in Tohoku149, 18 were amino acids, including Tyr, Leu, Ile, Met, Val, Lys, and Glu, or amino acid-related molecules, including *S*-adenosylmethionine, 2-oxoisopentanoate and 4-methyl-2-oxopentanoate (metabolized products of Met, Val, and Leu, respectively). The other seven metabolites included Ala, and Glu/Gln-related molecules. Our results were not consistent with those of Chiozza et al., who found that the concentrations of Asn, Gln, Glu, His, Pro, and Ser were lower in the aphid-resistant soybean leaves than aphid-susceptible leaves [33]. This difference might arise because of the investigated soybean strain and/or its developmental stage.

The distinct metabolite differences between the strains decreased with time, and both strains had similar profiles 6–24 h after aphid introduction. At 6 h, the concentrations of shikimate and *trans*-cinnamate, which are known precursors of phenylpropanoid synthesis [42], were significantly higher in Suzuyutaka leaves ($P = 0.0040$ and 0.0003, respectively) (Table 1 and Fig. 5) than Tohoku149 leaves. The phenylpropanoid family includes a large variety of secondary metabolites, such as lignin, suberin, tannin, flavonoids, alkaloids and phytoalexins [43], and these compounds function as mediators of various environmental factors, such as disease, pests, light and nutrition [42]. The concentration of tyramine, which is a known intermediate of secondary metabolites, was 4.5-times higher in Tohoku149 than in Suzuyutaka. Tyramine is located downstream of tyrosine and shikimate, which was present in high concentration in Tohoku149 before aphid introduction. These data suggest that rapid production of secondary metabolites might be implicated in aphid resistance. However, this needs to be confirmed by integrated analysis with both central and secondary metabolites.

The concentration of azelate was significantly higher in Tohoku149 than in Suzuyutaka at 24 and 48 h after aphid introduction. Azelate induces systemic immunity against the pathogen *Pseudomonas syringae* in *Arabidopsis*, and plays a role in signaling of systemic acquired resistance (SAR) [44,45]. To date the relationship between azelate and resistance has not been clarified, but our results show that azelate synthesis is caused by aphid infection.

The profiles of the metabolites that were expressed different in the two strains drastically changed 48 h after aphid introduction. Concentrations of the components of the TCA cycle (i.e., 2-oxoglutalate, malate, succinate, and citrate) increased in Tohoku149, while the concentrations of three glycolysis intermediates decreased. Increases in the concentrations of glucuronate and 4-aminobutanoate, which produce 2-oxoglutalate and succinate,

Table 1
Metabolites showing significantly different concentrations between Tohoku149 and Suzuyutaka.

KEGG ID	Metabolite	Tohoku149		Suzuyutaka		Fold change (Tohoku149/Suzuyutaka)	*p*-Value
		Average (fmole/mg)	S.E.	Average (fmole/mg)	S.E.		
(Before aphid inoculation)							
C00158	Citrate	384.24	105.06	11.22	11.22	34.26	0.0077*
C00082	Tyr	9.42	1.78	0.59	0.09	16.04	0.0011*
C00123	Leu	37.51	8.42	3.67	0.80	10.21	0.0039*
C00407	Ile	27.17	6.16	3.53	0.53	7.69	0.0051*
C00141	2-Oxoisopentanoate	0.45	0.07	0.06	0.06	7.38	0.0034*
C00233	4-Methyl-2-oxopentanoate	2.18	0.35	0.35	0.21	6.29	0.0022*
C00073	Met	0.61	0.10	0.13	0.03	4.59	0.0015*
C00183	Val	37.09	7.61	8.53	1.03	4.35	0.0059*
C02356	2-Aminobutanoate	1.48	0.21	0.43	0.01	3.46	0.0011*
C03618	*threo*-beta-methylaspartate	486.01	41.48	150.41	38.69	3.23	0.0004**
C00047	Lys	23.25	3.44	7.47	1.57	3.11	0.0031*
C00380	Cytosine	0.37	0.02	0.14	0.05	2.53	0.0018*
C00258	Glycerate	62.62	7.89	29.83	2.37	2.10	0.0041*
C00025	Glu	850.83	49.14	405.92	26.63	2.10	0.0000**
C00019	*S*-Adenosylmethionine	1.03	0.14	0.53	0.03	1.93	0.0080*
–	4-Oxopentanoate	2.20	0.47	0.00	0.00	–	0.0017*
C06104	Adipate	0.36	0.06	0.00	0.00	–	0.0003**
C02656	Pimelate	0.19	0.04	0.00	0.00	–	0.0012*
C02704	Methyl sulfate	0.57	0.05	0.82	0.04	0.70	0.0027*
C00186	Lactate	23.90	1.99	39.01	3.17	0.61	0.0038*
C00041	Ala	57.80	13.40	121.93	10.81	0.47	0.0058*
C01073	*N*-Acetyl-beta-alanine	0.21	0.05	0.44	0.03	0.47	0.0034*
C00263	Homoserine	0.27	0.08	0.63	0.06	0.43	0.0066*
C01047	*N*-gamma-ethylglutamine	1.16	0.18	3.70	0.41	0.31	0.0005**
C00624	*N*-Acetyl-L-glutamate	0.34	0.26	1.31	0.08	0.26	0.0071*
(6 h after aphid inoculation)							
C00483	Tyramine	1.30	0.26	0.29	0.09	4.46	0.0071*
C00199	Ribulose 5-phosphate	1.01	0.20	0.23	0.09	4.44	0.0076*
C00327	Citrulline	0.55	0.06	0.13	0.09	4.30	0.0039*
C00438	*N*-Carbamoyl-L-aspartate	0.33	0.09	0.00	0.00	–	0.0051*
C00423	*trans*-Cinnamate	14.63	0.92	27.87	1.92	0.52	0.0003**
C00493	Shikimate	3.72	1.04	13.61	2.24	0.27	0.0040*
C00431	5-Aminovalerate	0.56	0.38	4.70	0.76	0.12	0.0012*
(12 h after aphid inoculation)							
C00153	Nicotinamide	0.33	0.03	0.20	0.01	1.62	0.0097*
C00380	Cytosine	0.32	0.02	0.21	0.02	1.48	0.0067*
C01152	3-Methylhistidine	0.01	0.01	0.10	0.02	0.10	0.0051*
C01771	Crotonate	0.00	0.00	3.55	1.02	0.00	0.0081*
(24 h after aphid inoculation)							
C00153	Nicotinamide	0.38	0.03	0.10	0.06	3.90	0.0025*
C00148	Pro	25.28	2.65	11.02	1.99	2.30	0.0026*
C00158	Citrate	210.99	22.30	99.87	9.19	2.11	0.0017*
C02656	Pimelate	0.66	0.08	0.31	0.04	2.10	0.0046*
C08261	Azelate	0.32	0.03	0.16	0.02	2.04	0.0017*
C00025	Glu	587.22	40.19	392.63	38.45	1.50	0.0081*
C00114	Choline	103.77	6.97	70.90	38.45	1.46	0.0029*
C01042	*N*-Acetyl-L-aspartate	0.20	0.05	0.00	38.45	–	0.0046*
(48 h after aphid inoculation)							
C00191	Glucuronate	5.11	0.42	0.87	0.30	5.90	0.0000**
C00026	2-Oxoglutarate	63.52	5.53	12.50	5.27	5.08	0.0002**
C02504	2-Isopropylmalate	0.30	0.04	0.07	0.01	4.40	0.0009**
C05829	*N*-Carbamyl-L-glutamate	0.65	0.04	0.18	0.05	3.67	0.0001**
C00711	Malate	726.81	50.41	239.43	22.78	3.04	0.0000**
C00042	Succinate	16.00	2.17	5.73	0.79	2.79	0.0021*
C00387	Guanosine	1.11	0.13	0.48	0.11	2.32	0.0054*
C08261	Azelate	0.50	0.06	0.23	0.04	2.20	0.0039*
C00153	Nicotinamide	0.51	0.06	0.24	0.04	2.09	0.0058*
C00158	Citrate	300.62	17.92	155.87	23.05	1.93	0.0011*
C00380	Cytosine	0.38	0.02	0.20	0.02	1.88	0.0003**
C00025	Glu	600.27	17.19	320.74	34.22	1.87	0.0001**
C00334	4-Aminobutanoate	59.19	4.08	32.04	5.41	1.85	0.0039*
C06423	Octanoate	1.35	0.09	0.77	0.12	1.75	0.0056*
C00074	Phosphoenolpyruvate	0.81	0.28	0.66	0.16	1.24	0.0023*
C03618	*threo*-beta-methylaspartate	342.70	86.21	0.00	0.00	–	0.0041*
C11145	Methanesulfonate	1.13	0.32	0.00	0.00	–	0.0084*
C01073	*N*-Acetyl-beta-alanine	0.07	0.02	0.00	0.00	–	0.0076*
C00092	Glucose 6-phosphate	4.09	0.18	6.33	0.52	0.65	0.0037*
C00093	Glycerophosphate	0.73	0.07	2.07	0.22	0.35	0.0004**
C00197	3-Phosphoglycerate	0.11	0.06	0.62	0.07	0.18	0.0007**

Table 1 (*Continued*)

KEGG ID	Metabolite	Tohoku149		Suzuyutaka		Fold change (Tohoku149/Suzuyutaka)	*p*-Value
		Average (fmole/mg)	S.E.	Average (fmole/mg)	S.E.		
(96 h after aphid inoculation)							
C04501	*N*-Acetylglucosamine 1-phosphate	1.28	0.21	0.18	0.12	6.94	0.0019*
C01015	Hydroxyproline	0.36	0.04	0.06	0.06	6.17	0.0028*
C00483	Tyramine	1.12	0.06	0.34	0.10	3.30	0.0002**
C00082	Tyr	21.38	4.23	6.57	0.67	3.25	0.0086*
C00025	Glu	644.96	30.21	443.01	31.73	1.46	0.0017*

Note: S.E. denotes standard error. "–" indicate inifinit value.

* $p < 0.01$.

** $p < 0.001$.

confirmed elevation of the TCA cycle. It is likely that NADH and ATP are consumed to recover from damage caused by the aphids, including for scavenging reactive oxygen species, and repair of the cell walls and membrane. The activities for recovery from injury might be important traits for resistance, although we focused on the mechanism of aphid death.

3.5. Interaction between soybean leaves and aphids

Aphids depend on phloem sap, which contains few amino acids, as their sole energy source [46]. For amino acid supplementation, aphids have a unique system utilizing symbiotic bacteria [47]. Because of the large differences in the amino acid profiles of Tohoku149 and Suzuyutaka, we compared the amino acids profiles of the aphids 12 h after they were introduced to both strains (Fig. 6). Aphids kept under starvation were used as a negative control. Interestingly, the overall amino acid profile in the aphids on the Tohoku149 leaves was similar to that for the aphids under starvation. By contrast, that for the aphids on the Suzuyutaka leaves was different, which suggests the soybean amino acids affected these aphids. Assuming that this aphid has the same amino acid metabolism pathways as the pea aphid, *Acyrthosiphon pisum*, and its symbiotic bacterium *Buchnera aphidicola*, nine amino acids (His, Trp, Lys, Arg, Val, Ile, Leu, Thr, Met, and Phe) can be synthesized from other amino acids in the phloem [48]. However, Trp was present at significantly higher levels in aphids on Tohoku149 leaves than on Suzuyutaka leaves ($P = 0.029$). These data suggest that the amino acid metabolism in this aphid is different from the model aphid, or that other metabolites from the soybean interact with the aphid metabolism. This might

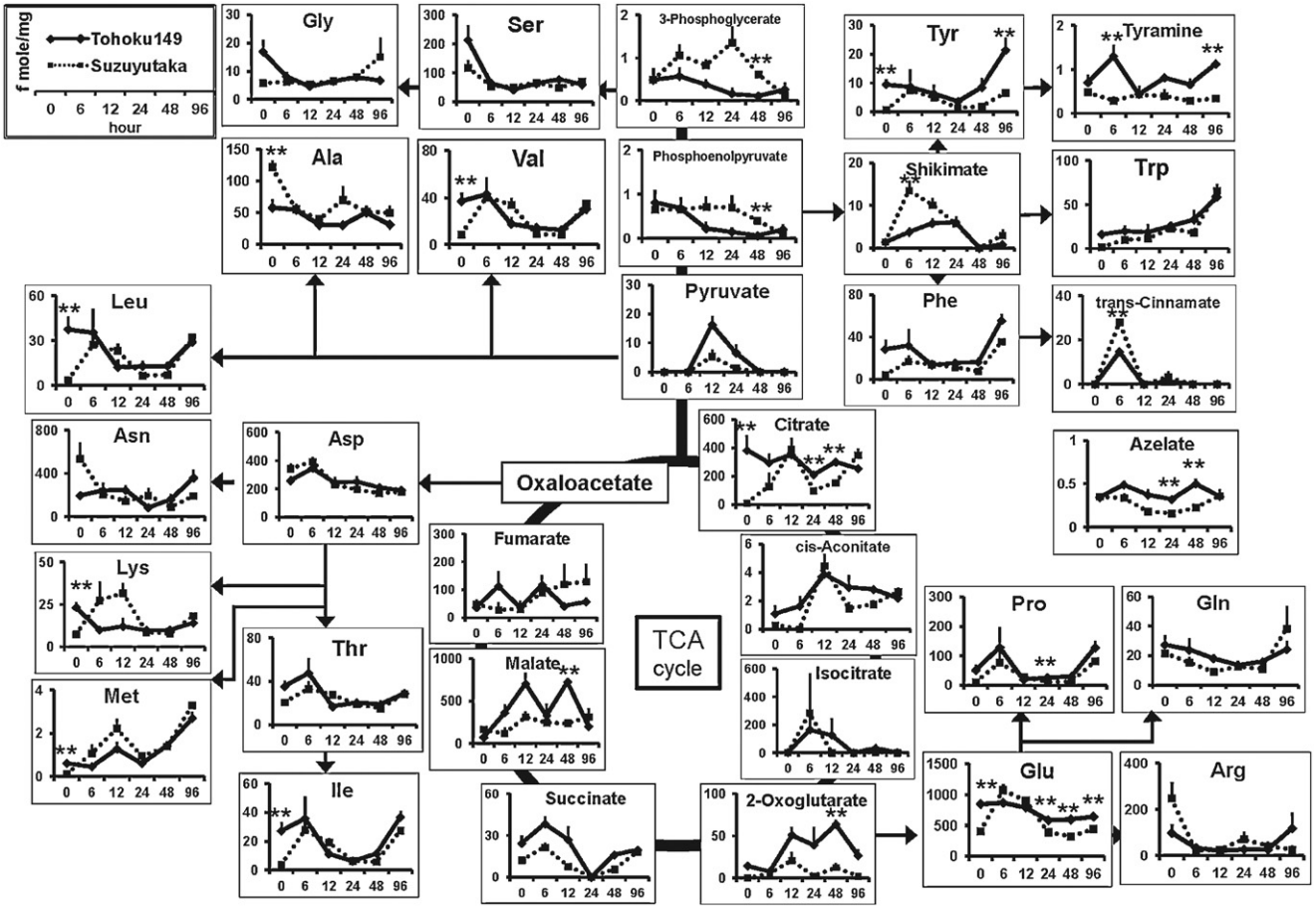

Fig. 5. Changes in the level of representative primary metabolites after aphid introduction. Data for the metabolite per mg of soybean leaves (wet weight) at 0–96 h after aphid introduction are plotted. Solid and dashed lines indicate the metabolite concentrations in Tohoku 149 ($n = 5$) and Suzuyutaka ($n = 5$), respectively. The data points are mean values and the error bars indicate the standard error. Asterisk (**) denotes statistical significance with $P < 0.01$

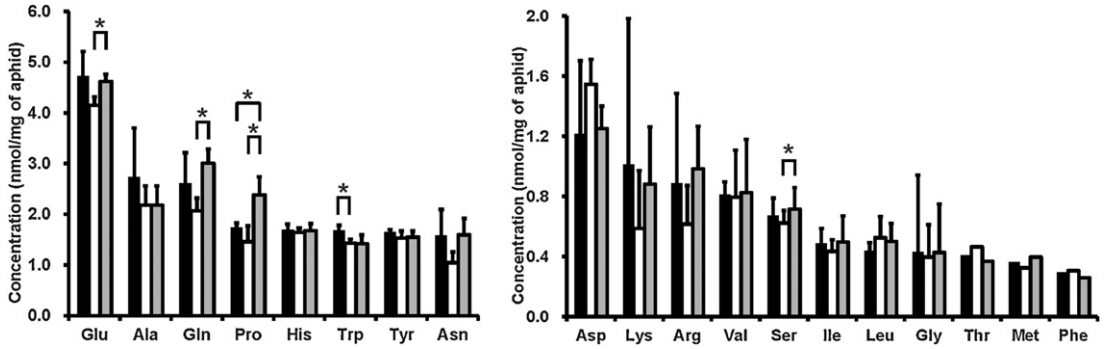

Fig. 6. Free amino acid contents in aphids that stayed on the Tohoku149 leaves (black) and Suzuyutaka leaves (white), and under starvation (gray) at 12 h. Amino acids in the insect bodies were measured in triplicate (mean ± S.E.). Cysteine was not detected. Asterisk (*) denotes statistical significance with $P < 0.05$. Error bars of threonine, methionine, and phenylalanine were omitted because S.E. values were too small to depict.

provide a clue to understanding the aphid resistance mechanism.

3.6. Limitations and perspective

We have validated and described metabolite profiling of soybean leaves in response to aphid introduction. To our knowledge, this is the first report of metabolomic analysis of both soybeans and aphids. Li et al. investigated gene-expression differences between resistant and susceptible soybean strains [24]. Integrated analysis of metabolomic and transcriptomic data, and profiling of secondary metabolomic profiles will contribute to the understanding of the aphid-resistance mechanism. Although CE–TOFMS is one of the best tools to analyze metabolites, it can only profile charged and hydrophilic metabolites [49]. To expand the range of metabolites that could be profiled, in future research we will conduct non-targeted analysis to detect all possible peaks and identify their precursor ions using other technologies. These techniques could include tandem-MS and/or combined analysis with other types of hyphened-MS methods, such as liquid chromatography–MS and gas chromatography/MS, to profile non-polar and volatile metabolites [50–52].

In this initial study, we only compared two strains, aphid-resistant and -susceptible, to simplify the experimental design. Study of additional strains would be useful for evaluating the specifics of the observed metabolomic features, and field experiments would be useful for considering many related factors. Identification of the resistance mechanism at the molecular level will facilitate discovery of new markers to screen for resistant strains, and to reduce the use of insecticides.

3.7. Concluding remarks

We characterized the aphid-resistance of two soybean strains, Tohoku149 and Suzuyutaka, and analyzed the metabolomic profiles of these strains to understand the mechanism underling aphid resistance. Aphid survival and settlement rates demonstrated that Tohoku149 has strong aphid resistance compared to Suzuyutaka. Comparative metabolomic analyses revealed that the concentrations of many metabolites, such as amino acids and their related molecules, were higher in Tohoku149 than in Suzuyutaka. After aphid introduction, these differences were dramatically reduced at 6–24 h, and key molecules leading to secondary metabolites were present. After 48 h, metabolites related to energy metabolism increased in the aphid-resistant strain, presumably because recovery from damage caused by aphid feeding is critical. Differences in the amino acids in the soybean leaves influenced the free amino acids found in the aphids, which might be implicated in aphid resistance.

Acknowledgements

We would like to thank Yamagata Prefectural Rice Breeding and Crop Science Experiment Station for providing the soybean seeds, and Lowland Farming Research Division, Tohoku Agricultural Research Center for their valuable advice regarding the character of Tohoku149. This work was supported by research funds from Yamagata Prefectural Government and Tsuruoka City.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2013.02.036.

References

[1] G. Hartman, E. West, T. Herman, Food Secur. 3 (2011) 5.
[2] K.F. Harris, Annu. Rev. Phytopathol. 19 (1981) 391.
[3] R.E. Down, A.M.R. Gatehouse, W.D.O. Hamilton, J.A. Gatehouse, J. Insect Physiol. 42 (1996) 1035.
[4] S.E. Jandricic, S.P. Wraight, K.C. Bennett, J.P. Sanderson, Environ. Entomol. 39 (2010) 1631.
[5] T. Saito, K. Ueno, K. Takeda, K. Oda, H. Honda, Annu. Rept. Plant Prot. North Jpn. 52 (2001) 178.
[6] K. Ueno, H. Takatori, T. Saito, Annu. Rept. Plant Prot. North Jpn. 52 (2001) 183.
[7] T. Nagano, Y. Umetsu, N. Hoshi, T. Kidokoro, Annu. Rept. Plant Prot. North Jpn. 52 (2001) 175.
[8] T. Nagano, Y. Umetsu, K. Onodera, T. Kidokoro, Annu. Rept. Plant Prot. North Jpn. 52 (2001) 172.
[9] T. Ono, J. Oyama, T. Kidokoro, Annu. Rept. Plant Prot. North Jpn. 57 (2006) 148.
[10] T. Nakata, Appl. Entomol. Zool. 30 (1995) 129.
[11] T.H. Schuler, G.M. Poppy, B.R. Kerry, I. Denholm, Trends Biotechnol. 16 (1998) 168.
[12] S. Ohnishi, N. Miyake, T. Takeuchi, F. Kousaka, S. Hiura, O. Kanehira, M. Saito, T. Sayama, A. Higashi, M. Ishimoto, Y. Tanaka, S. Fujita, Breed. Sci. 61 (2012) 618.
[13] Y. Li, C.B. Hill, G.L. Hartman, J. Econ. Entomol. 97 (2004) 1106.
[14] M. Rossi, F.L. Goggin, S.B. Milligan, I. Kaloshian, D.E. Ullman, V.M. Williamson, Proc. Natl. Acad. Sci. U.S.A. 95 (1998) 9750.
[15] X.M. Liu, C.M. Smith, B.R. Friebe, B.S. Gill, Crop Sci. 45 (2005) 2273.
[16] S. Mittal, L.S. Dahleen, D. Mornhinweg, Crop Sci. 48 (2008) 1452.
[17] Y. Brotman, L. Silberstein, I. Kovalski, C. Perin, C. Dogimont, M. Pitrat, J. Klingler, A. Thompson, R. Perl-Treves, Theor. Appl. Genet. 104 (2002) 1055.
[18] J. Klingler, R. Creasy, L. Gao, R.M. Nair, A.S. Calix, H.S. Jacob, O.R. Edwards, K.B. Singh, Plant Physiol. 137 (2005) 1445.
[19] Y.-S. So, H. Ji, J. Brewbaker, Euphytica 172 (2010) 373.
[20] W. Akbar, A.T. Showler, J.M. Beuzelin, T.E. Reagan, K.A. Gravois, Ann. Entomol. Soc. Am. 104 (2011) 699.
[21] C.B. Hill, Y. Li, G.L. Hartman, Crop Sci. 44 (2004) 98.
[22] Y. Li, C.B. Hill, S.R. Carlson, B.W. Diers, G.L. Hartman, Mol. Breed. 19 (2007) 25.
[23] C. Hill, A. Chirumamilla, G. Hartman, Euphytica 186 (2012) 635.
[24] Y. Li, J. Zou, M. Li, D.D. Bilgin, L.O. Vodkin, G.L. Hartman, S.J. Clough, New Phytol. 179 (2008) 185.
[25] I. Morkunas, V. Mai, B. Gabryś, Acta Physiol. Plant. 33 (2011) 2057.

[26] W. Wu, Q. Zhang, Y. Zhu, H.-M. Lam, Z. Cai, D. Guo, J. Agric. Food Chem. 56 (2008) 11132.
[27] S. Komatsu, A. Yamamoto, T. Nakamura, M.-Z. Nouri, Y. Nanjo, K. Nishizawa, K. Furukawa, J. Proteome Res. 10 (2011) 3993.
[28] J. Cheng, C. Yuan, T.L. Graham, Phytochemistry 72 (2011) 875.
[29] N. Benkeblia, T. Shinano, M. Osaki, Metabolomics 3 (2007) 297.
[30] M. Sugimoto, H. Goto, K. Otomo, M. Ito, H. Onuma, A. Suzuki, M. Sugawara, S. Abe, M. Tomita, T. Soga, J. Agric. Food Chem. 58 (2010) 8418.
[31] N. Takuji, O. Keiki, B. Noureddine, W. Jun, W. Toshihiro, M. Hideyuki, U. Hirofumi, K. Setsuko, S. Takuro, Genetics, Genomics, and Breeding of Soybean, Science Publishers, Enfield, NH, USA, 2010, p. 313.
[32] C. Mensah, C. DiFonzo, R.L. Nelson, D. Wang, Crop Sci. 45 (2005) 2228.
[33] M.V. Chiozza, M.E. O'Neal, G.C. MacIntosh, Environ. Entomol. 39 (2010) 856.
[34] C.B. Hill, Y. Li, G.L. Hartman, Crop Sci. 46 (2006) 1601.
[35] C.B. Hill, Y. Li, G.L. Hartman, Crop Sci. 46 (2006) 1606.
[36] C.B. Hill, K.-S. Kim, L. Crull, B.W. Diers, G.L. Hartman, Crop Sci. 49 (2009) 1193.
[37] G. Zhang, C. Gu, D. Wang, Theor. Appl. Genet. 120 (2010) 1183.
[38] G. Zhang, C. Gu, D. Wang, Theor. Appl. Genet. 118 (2009) 473.
[39] O. Takahashi, K.-I. Honda, S. Kawabe, Appl. Entomol. Zool. 37 (2002) 577.
[40] J. Diaz-Montano, J.C. Reese, J. Louis, L.R. Campbell, W.T. Schapaugh, J. Econ. Entomol. 100 (2007) 984.
[41] S.-J. Park, Y. Huang, P. Ayoubi, Planta 223 (2006) 932.
[42] T. Vogt, Mol. Plant 3 (2010) 2.
[43] K.M. Herrmann, Plant Physiol. 107 (1995) 7.
[44] H.W. Jung, T.J. Tschaplinski, L. Wang, J. Glazebrook, J.T. Greenberg, Science 324 (2009) 89.
[45] J.E. Parker, Sci. Signal. 2 (2009) 31.
[46] A.F.G. Dixon, Annu. Rev. Ecol. Syst. 8 (1977) 329.
[47] A.E. Douglas, Annu. Rev. Entomol. 43 (1998) 17.
[48] The International Aphid Genomics Consortium, PLoS Biol. 8 (2010) e1000313.
[49] M.R.N. Monton, T. Soga, J. Chromatogr. A 1168 (2007) 237.
[50] H.G. Gika, G.A. Theodoridis, I.D. Wilson, J. Sep. Sci. 31 (2008) 1598.
[51] R. Ramautar, E. Nevedomskaya, O.A. Mayboroda, A.M. Deelder, I.D. Wilson, H.G. Gika, G.A. Theodoridis, G.W. Somsen, G.J. de Jong, Mol. Biosyst. 7 (2011) 194.
[52] G. Graça, B.J. Goodfellow, A.S. Barros, S. Diaz, I.F. Duarte, K. Spagou, K. Veselkov, E.J. Want, J.C. Lindon, I.M. Carreira, E. Galhano, C. Pita, A.M. Gil, Mol. Biosyst. 8 (2012) 1243.