JOURNAL OF
AGRICULTURAL AND
FOOD CHEMISTRY

Metabolite Profiling of Mizuna (*Brassica rapa* L. var. Nipponsinica) To Evaluate the Effects of Organic Matter Amendments

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Supporting Information

ABSTRACT: Organic matter amendment is an essential agricultural protocol to improve soil function and carbon sequestration. However, the effect of organic matter amendments on crop quality has not been well-defined. This study applied gas chromatography−mass spectrometry to investigate the metabolite profiling of mizuna (*Brassica rapa* L. var. Nipponsinica) with different organic matter amendments with respect to quality and quantity. Principal component analysis showed that 33.4, 15.6, and 6.6% of the total variance was attributable to the plant N concentration, fast-release organic fertilizer (fish cake), chicken droppings), and rapeseed cake), and manure application (fresh and dried), respectively. The peak areas of 18 and 15 compounds were significantly altered under organic fertilizer and manure amendment, respectively, compared with pure chemical fertilizer amendment. The compounds altered with manure amendment were similar to those reported in previous studies using other species. This study is the first to show clear metabolic alterations in plants through the amendment of fast-release organic fertilizer. Mizuna is a unique plant species that responds to both organic fertilizer and manure. These observations are useful to clarify the effect of organic matter amendment and quality control in farming systems using organic matter.

KEYWORDS: *Brassica rapa L., fast-release organic fertilizer, cattle manure, nitrogen supply, GC-MS*

INTRODUCTION

After the invention of artificial nitrogen (N) fixation in the early 20th century, the use of organic matter amendment in organic farming and on agricultural commodities grown has been substituted with chemical fertilization; however, there has been a resurgence in the use of organic matter amendment, reflecting the increasing demand for sustainable food production. Although organic matter amendment has been recommended for improving soil physical properties and microbial diversity, the mitigation of CO_2 emissions, the effects of recycling nutrients,[1,2] and the quality of agricultural crops grown under organic matter amendment have not been characterized.

For many years, researchers have studied whether organic matter amendment and organic farming enhance or reduce the quality of agricultural commodities,[3−5] but the results have been inconsistent and no consensus has been obtained. Particularly, the effects of organic matter amendment have been evaluated on parameters, such as major metabolites and minerals, in research concerning constituents of crops.[6−9]

A more comprehensive approach for the evaluation of crop constituents has been conducted through the application of "metabolomics" techniques in recent years. Metabolomics is the identification and quantification of a set of metabolites involved in a biological system, which provides a reasonable approach to the physiological characterization of plants. Metabolites are the end products of cellular regulatory processes; thus, metabolite levels are indicative of the ultimate response of biological systems to genetic or environmental changes.[10]

Various tools have been employed in metabolomics studies; however, the technique of gas chromatography−mass spectrometry (GC-MS) is suitable for the unbiased profiling of primary metabolites in plants.[11] GC-MS-based metabolic profiling is a convenient and powerful method for distinguishing silent plant phenotypes among mutants[12] and clarifying physiological mechanisms, such as stress tolerances[13] and diurnal patterns.[14] Primary metabolites directly influence the growth and reproduction of plants. Particularly, polar primary metabolites, such as sugars, amino acids, and organic acids are directly affected by factors, including N absorption and carbon assimilation, via pathways such as the TCA cycle, photosynthesis, glycolysis, and amino acid biosynthesis. In addition, these compounds are also important in food quality associated with human taste parameters.

Several studies to characterize the growth of organic agricultural commodities have been conducted on cereals, such as wheat[15,16] and maize,[17] in field conditions using GC-MS-based metabolite profiling; however, only slight differences were observed between organic and conventional methods. These results demonstrated that many factors affect plant metabolites, for example, environmental influences, cultivar, and plant growth. In addition, a high degree of homeostasis of metabolites in the final seed set, independent of the growing regime, has also been suggested.[16]

In general, the N supply is a major environmental factor regulating plant components and is closely associated with crop

Received: September 11, 2012
Revised: December 16, 2012
Accepted: December 17, 2012
Published: December 17, 2012

Table 1. Pot Experiment (Pot Area = 0.02 m^2, Volume = 3.2 L)

(A) Dose of Applications

		N		P	K	manure
unit		total N (g pot^{-1})		P_2O_5 (g pot^{-1})	K_2O (g pot^{-1})	fresh manure (kg pot^{-1})
amount	L0	L1	L2	0.2	0.24	0.06
	0	0.09	0.18			

(B) Materials of Application

		materials			
treatment	ID	N	P	K	manure
chemical (C)	C-L0	ammonium sulfate	superphosphate	potassium sulfate	
	C-L1				
	C-L2				
organic (O)	Of-L1	fish cake			
	Of-L2				
	Oc-L1	chicken droppings	guano	natural potassium sulfate	
	Oc-L2				
	Or-L1	rapeseed cake			
	Or-L2				
chemical (N) + manure (C+M)	C+Md	ammonium sulfate (L1)			dried manure
	C+Mf				fresh manure

quality. Comprehensive studies using GC-MS-based methodology[18,19] on spinach (*Spinacia oleracea* L.) grown with different levels and sources of N showed that both treatments significantly altered leaf metabolite profiles, which is a process not solely attributable to rates of N uptake and changes in dry weight. Furthermore, a comprehensive analysis of the metabolite compositions of radish (*Raphanus sativus* L.) leaves and roots using a split-plot design involving applied inorganic N and manure rates was conducted to evaluate the effects of manure on the quality of this crop,[20] and on the basis of the results of the principal component analysis (PCA), these studies demonstrated that metabolites responded strongly to the amount of manure applied, irrespective of the effects of chemical N fertilizer application. In addition, fast-release organic fertilizers are typically used in organic farming, with or without manure. The N fertilizer effects of organic fertilizers differ from those of manure, which has little N fertilizer effect, as crops absorb the rapidly decomposed N in fast-release organic fertilizers. In the present study, we attempted to characterize the influence of fast-release organic fertilizer on different plants types and evaluate the effects of fast-release organic fertilizers and manure on metabolite composition. Moreover, organic fertilizers have diverse origins and properties (pH, C/N, and nutrient concentration, see Table S3 in the Supporting Information); thus, it has been proposed that these substances contain a different nutrient supply and affect microorganism activity in the soil. Thus, it is important to determine the differences among organic fertilizers produced from different origins. To this end, we prepared three types of fast-release organic fertilizers: fish cake, chicken droppings, and rapeseed cake. Moreover, although farmers typically use fresh manure, we also determined whether dried manure reduces the effects of microorganisms.

We comprehensively analyzed the metabolite compositions of mizuna (*Brassica rapa* L. var. Nipposinica) shoots using a GC-MS-based methodology to determine whether plant metabolism was affected differently through the use of amendment of organic fertilizers or manure compared with chemical fertilizers. Mizuna is a traditional Japanese leafy vegetable that belongs to Brassicaceae. We observed that some samples of mizuna grown under organic farming tasted different from those grown conventionally. Organically grown mizuna had a bitter and rich taste and a specific metabolite composition, which was affected after the addition of organic materials or other factors. To strictly control environmental factors and nutrient supply, a greenhouse pot experiment was conducted using high and low N input levels for each material under chemical and organic treatments to evaluate the effects of fast-release organic fertilizers on the metabolite compositions. Our previous data suggested that N status highly influenced metabolite compositions in plants;[18,20] therefore, we employed a method to suitably remove the effects of N status on the compounds in mizuna. Analysis of covariance (ANCOVA) was used to evaluate the effects of organic fertilizers and manures under conditions in which the influence of N contents of plants was removed.

■ MATERIALS AND METHODS

Plant Material. In this study, we used mizuna (*B. rapa* L. var. Nipposinica), a Japanese leafy vegetable from the Brassicaceae family, that has traditionally been used as an ingredient in Japanese cooking.

In 2011, the cultivation of mizuna was conducted in a greenhouse at the NARO Hokkaido Agricultural Research Center (NARO/HARC), Japan. Melanaquand soil was obtained from a field at NARO/HARC. The soil pH was adjusted to 5.7 using $CaCO_3$. For pot cultivation, the soil and vermiculite were mixed at 5:3 (v/v) and used to fill a 1/5000a Wagner pot (3.2 L). The soil properties are shown in Table S1 in the Supporting Information.

Three treatments were employed in this study: chemical fertilization (C), organic fertilization (O), and chemical (nitrogen) fertilization plus manure amendment (C+M). The O treatment was further divided into three treatments depending on the variety of the organic fertilizer: fish cake (Of), chicken droppings (Oc), and rapeseed cake (Or), as these organic fertilizers are considered to be fast-release fertilizers. High and low N input levels (L1 and L2 of 0.09 and 0.18 g pot^{-1}, respectively) were used for the C and each O treatment. As a control, a no-N fertilizer treatment was added to the C treatment (C-L0). Ammonium sulfate was used for the C treatment. For the C+M treatment, two types of dairy cattle manure (dried and fresh) were used, which were produced at NARO/HARC and composted over 4 months with turning. Dry manure was produced in 2006 (dried in a

Table 2. Shoot Fresh Weight, Dry Matter Ratio, N Absorption, and Total N, Total P, Total K, and NO_3^- Concentrations in Mizuna Shoots Grown with Chemical, Organic, and Chemical (Nitrogen) plus Manure Treatments[a]

treatment	shoot FW (g $plant^{-1}$)	dry matter ratio	N absorption (mg $plant^{-1}$)	total N conc. (g kg^{-1} DW)	total P conc. (g kg^{-1} DW)	total K conc. (g kg^{-1} DW)	NO_3 (g kg^{-1} FW)
chemical							
C-L0	18.0 ± 0.62 e	0.133 ± 0.003 a	28.3 ± 1.05 g	11.8 ± 0.14 d	2.10 ± 0.15 bc	31.0 ± 1.66 d	nd
C-L1	33.9 ± 0.76 c	0.101 ± 0.004 bc	60.8 ± 1.19 cde	17.9 ± 0.70 bc	2.14 ± 0.22 bc	47.8 ± 4.89 abc	0.46 ± 0.09 cd
C-L2	42.4 ± 1.59 b	0.090 ± 0.003 c	87.6 ± 2.99 a	23.1 ± 1.32 a	2.09 ± 0.15 c	55.0 ± 1.58 ab	1.16 ± 0.20 ab
organic							
Of-L1 (fish cake)	27.7 ± 0.31 d	0.104 ± 0.007 bc	49.9 ± 1.50 ef	17.6 ± 1.18 bc	1.96 ± 0.11 c	46.0 ± 4.54 abcd	0.45 ± 0.11 cd
Of-L2	37.7 ± 0.70 bc	0.094 ± 0.003 bc	72.1 ± 0.96 b	20.4 ± 0.72 abc	2.26 ± 0.10 bc	48.9 ± 3.78 abc	0.87 ± 0.17 abc
Oc-L1 (chicken droppings)	25.1 ± 0.85 d	0.114 ± 0.004 ab	46.5 ± 2.09 f	16.4 ± 1.46 cd	1.78 ± 0.13 c	38.5 ± 1.70 cd	0.31 ± 0.07 cd
Oc-L2	35.3 ± 0.71 c	0.096 ± 0.005 bc	59.1 ± 1.82 de	17.5 ± 0.32 bc	2.19 ± 0.03 bc	48.3 ± 3.18 abc	0.56 ± 0.10 cd
Or-L1 (rapeseed cake)	26.6 ± 0.74 d	0.108 ± 0.006 bc	50.4 ± 2.89 ef	17.8 ± 1.62 bc	2.01 ± 0.09 c	44.8 ± 1.70 bcd	0.36 ± 0.14 cd
Or-L2	35.1 ± 0.99 c	0.091 ± 0.004 c	70.4 ± 3.92 bc	22.2 ± 0.90 ab	2.11 ± 0.06 bc	57.0 ± 1.99 ab	1.18 ± 0.12 a
chemical (N) + manure							
C+Md (dried manure)	41.0 ± 1.68 b	0.098 ± 0.004 bc	69.5 ± 2.73 bcd	17.4 ± 0.83 bc	2.71 ± 0.12 ab	49.3 ± 4.02 abc	0.20 ± 0.09 d
C+Mf (fresh manure)	54.6 ± 1.61 a	0.088 ± 0.003 c	96.5 ± 1.74 a	20.1 ± 0.46 abc	3.04 ± 0.11 a	60.1 ± 2.85 a	0.58 ± 0.09 bcd
mean							
chemical (except C-L0)	38.1 ± 1.80	0.095 ± 0.003	74.2 ± 5.28	20.5 ± 1.20	2.11 ± 0.12	51.4 ± 2.74	0.81 ± 0.17
organic	31.3 ± 1.06	0.101 ± 0.002	58.1 ± 2.28	18.6 ± 0.58	2.05 ± 0.05	47.3 ± 1.59	0.62 ± 0.08
chemical (N) + manure	47.8 ± 2.79	0.093 ± 0.003	83.0 ± 5.32	18.7 ± 0.67	2.88 ± 0.10	54.7 ± 3.06	0.39 ± 0.09
significance							
chemical (except C-L0) vs organic	**	NS	**	NS	NS	NS	NS
chemical vs chemical (N) + manure	*	NS	NS	NS	**	NS	*

[a] Values (mean ± SE) within a column followed by different letters were significantly different (Tukey' s *t* test; $P < 0.05$). Significance, Student's *t* test: $P < 0.05$. FW, fresh weight; DW, dry weight; nd, not determined.

greenhouse for a week), and fresh manure was produced in 2009. Dried (C+Md) and fresh (C+Mf) manures were used at 0.06 kg pot^{-1}, and 0.09 g N pot^{-1} of ammonium sulfate was used. Phosphorus (P) was applied uniformly to the C treatment 0.2 g P_2O_5 pot^{-1} using superphosphate, and guano (certified as organic fertilizer) was used for the O treatment. Potassium (K) was applied to the C treatment uniformly at 0.24 g K_2O pot^{-1} using potassium sulfate, and natural potassium sulfate (certified as organic fertilizer) was applied to the O treatment. No P or K fertilizer was applied to the C+M treatment, as the manure contained sufficient amounts of P and K (Supporting Information, Table S2). The amounts of the organic fertilizers were calculated using the guaranteed component rates of each fertilizer (Supporting Information, Table S3). Details concerning the materials applied for each treatment are shown in Table 1.

There were four replicate pots per treatment. On July 23, 2010, four mizuna seeds (cv. Kyo-mizore) were sown per pot. After germination, two plants per pot were generated through thinning for further growth. On August 25, 33 days after sowing, the shoot of each plant was harvested. After the fresh weight was determined, the tissue was frozen and lyophilized for subsequent analysis of primary metabolites, minerals, and anions.

Determination of Mineral Elements and Anions. The mineral element and anion concentrations in the lyophilized samples were determined. The total N and P were analyzed using a segmented-flow analysis system (QuAAtro2-HR, BL-TEC, Japan) according to the manufacturer's instructions after Kjeldahl digestion: briefly, the shoots were digested using concentrated H_2SO_4 containing salicylate (33.4 g L^{-1}), followed by the addition of H_2O_2 at 300 °C. The amount of NO_3-N was included in the amount of total N. The K concentrations was determined using ICP-AES (SPS7800, Seiko Instruments, Japan) after Kjeldahl digestion. Nitrate anions (NO_3^-) were analyzed using ion chromatography with an IonPac column (ICSep AN300, Tokyo Chemical Industry, Japan).

Metabolite Profiling. The metabolite analysis was conducted according to the methods of Roessner et al.[11] and Okazaki et al.[20] Lyophilized shoot tissue was mashed in a 2 mL tube using a ball mill (MM400, Restch, Haan, Germany). Subsequently, 0.8 mL of ice-cold extraction solution (3:1:1 of methanol/chloroform/water), including an internal standard (ribitol at 0.64 μg mL^{-1}), was added to the mashed tissue, and the slurry was mixed for 5 min using a microtube mixer. Approximately 160 μL of distilled water was added to the extraction solution to separate the polar and nonpolar phases. After centrifugation (at 14000 rpm for 5 min), the upper methanol/water phase was transferred to a glass vial containing 0.2 mL of insert, concentrated using a centrifugal evaporator (CVE-200D; EYELA, Tokyo, Japan) and fully desiccated using phosphorus pentoxide in vacuo, followed by derivatization for GC-MS analysis. The samples were derivatized using methoxyamine hydrochloride (90 min at 40 °C) and *N*-methyl-*N*-trifluoroacetamide (MSTFA), containing a retention time standard mixture (*n*-decane, *n*-dodecane, *n*-pentadecane, *n*-octadecane, *n*-nonadecane, *n*-docosane, *n*-octacosane, *n*-dotriacontane, and *n*-hexatriacontane at 0.029% v/v in pyridine) for 30 min at 40 °C in a glovebox filled with Ar gas. The samples (1 μL) were injected into the Agilent GC 6890 in the splitless mode. The GC was performed on an Rtx 5Sil MS with an integrated guard column (30 m and 0.25 μm film; Restek GmbH, Bad Homburg, Germany). The column end was introduced into a GCmate-II sector mass spectrometer (JEOL, Tokyo, Japan). The mass spectra were recorded at two scans per second using an *m*/*z* 50−600 scanning range. The metabolites were identified using AMDIS software (http://chemdata.nist.gov/mass-spc/amdis/). Before statistical analysis, the data were normalized using the peak area of the internal standard, ribitol.

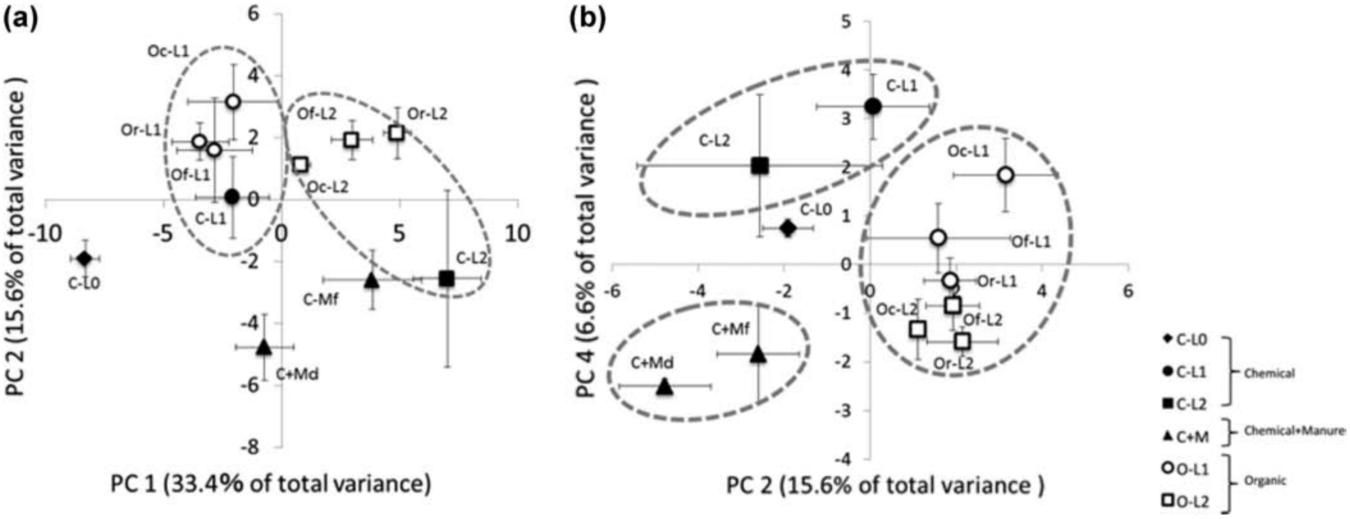

Figure 1. Principal component analysis (PCA) of metabolites in shoot extracts of mizuna. Scores of PCA are presented on the basis of combinations of PC1 and PC2 (a) and PC2 and PC4 (b). Each group was calculated from four samples. Error bar represents standard error.

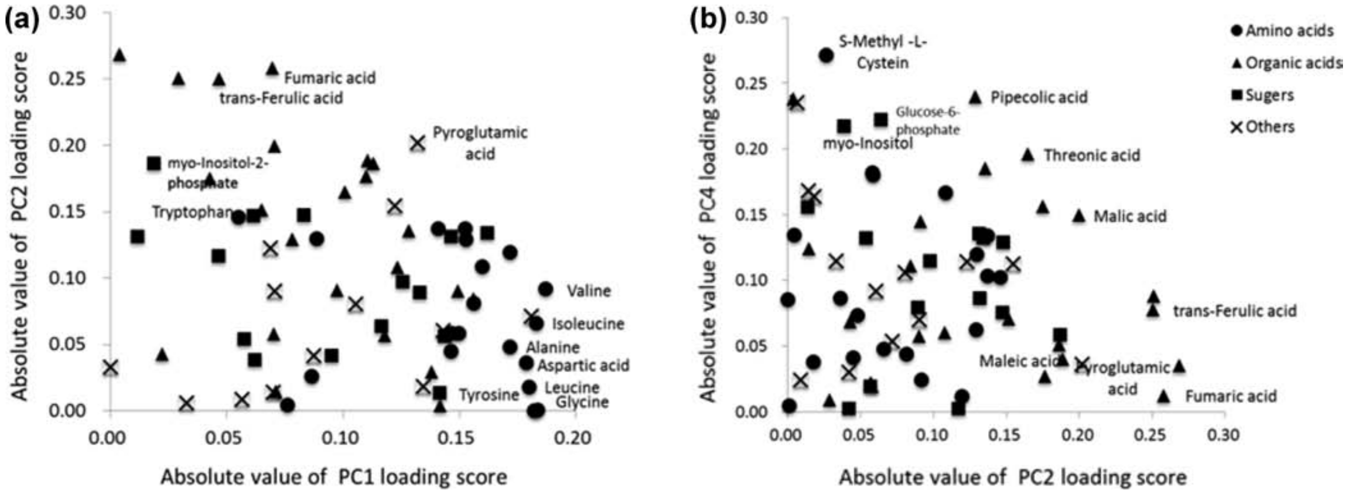

Figure 2. Absolute values of the compound loading scores for the first principal component (PC1) against the second (PC2) (a) and for PC2 against the fourth (PC4) (b) drawn from the principal component analysis shown in Figure 1. Higher absolute values indicate a greater contribution of the principal components for nitrogen content (PC1), differences between chemical and organic treatments (PC2), or effects of manure amendment (PC4).

Multivariate statistics were performed using JMP 9.0.0 (SAS, Cary, NC, USA).

■ RESULTS

Plant Growth and N, P, and K Contents. At harvest, the shoot fresh weight varied according to N input levels and applied materials (Table 2). The total N concentration reflected N input levels, but showed slight differences, except for the treatment of no-N input (C-L0). Mizuna grown with applied fresh manure (C+Mf) showed the highest shoot fresh weight, N absorption, and total P and K concentrations (Table 2). Other mineral elements and anions varied according to the treatments (Data not shown). Overall, the growth and nutrient condition of the samples reflected the types and amounts of applied materials. As our aim was to investigate the effects of organic matter amendment on the metabolites separately from the other factors, particularly the plant N content, we therefore considered these samples suitable for further experimentation.

Profiling of the Identified Metabolites. A total of 71 compounds, including 20 amino acids, 23 organic acids, 15 sugars, and 13 other compounds, were identified using GC-MS in the mizuna shoot extracts. Each peak area was subjected to PCA. The plots of the first and second (PC1 and PC2, respectively; Figure 1a) and second and fourth (PC4; Figure 1b) principal component scores revealed differences in the metabolite profiles, and the results clearly corresponded to differences in N levels, forming distinct clusters corresponding to the applied materials (Figure 1). PC1 accounted for 33.4% of the total variance, and this component affected the N input levels. This observation was confirmed through a significant correlation ($P < 0.01$) between the PC1 score and the N concentration of the mizuna shoots (Figure S1, Supporting Information). The strong positive relationship indicated a close interaction between the metabolite profiles and plant N content. PC2 contributed 15.6% of the total variance and discriminated between the C and O treatments. PC4 contributed 6.6% of the total variance and clearly separated the C+M from the C treatments, but did not clearly discriminate between the C+M and O treatments.

The contributions of each compound were reflected in the absolute values of PC compound loading scores. The effects of N content (PC1) were plotted against the differences between chemical and organic treatments (PC2), and PC2 was plotted against the effects of manure amendment (PC4) (Figure 2). Most amino acids showed higher absolute values for the PC1 loading scores, indicating that the N contents strongly affected the amino acid concentration. Some compounds showed high PC2 loading scores, indicating that the difference between the C and O treatments affected these compounds (Figure 2a). Certain compounds showed high absolute values for the PC4 loading scores, indicating that manure amendment partially affected these compounds (Figure 2b).

PCA demonstrated differences in the composition of detected metabolites between the C, O, and C+M treatments. Therefore, we conducted ANCOVA to select the metabolites significantly affected by the fast-release organic fertilizers compared with chemical fertilizers and manure amendments, that is, the difference between (1) C and O and (2) C and C+M. In addition, the N concentration was set as a predictor variable in the ANCOVA to remove the influence of the N concentration (the main factor of PC1) on each compound.

ANCOVA was performed on the normalized peak areas of 71 identified compounds using the N concentration and the O or C treatments as predictor variables, except for the C-L0 treatment, to identify the candidate compounds that were significantly affected after the O and C treatments. There were no significant interactions in 68 compounds, and 47 of these differed significantly according to N concentration and/or fertilization (Figure 3). Nine compounds showed significant differences according to fertilization alone, and nine compounds were significantly affected by the N concentration and fertilization; 29 compounds showed significant responses only to N concentration. Groups of similar metabolites showed coordinated differences between the C and O treatments. Five amino acids (threonine, asparagine, glutamine, serine, and *S*-methyl-L-cysteine) and two polyamines (spermidine and putrescine) showed significantly lower concentrations under O treatments compared with the C treatment; tryptophan was the only amino acid with a significantly higher concentration under O treatment. Five organic acids showed a significant difference between the C and O treatments: benzoic, threonic, and pipecolic acids showed significantly lower concentrations, whereas maleic and fumaric acids exhibited higher concentrations under O treatment.

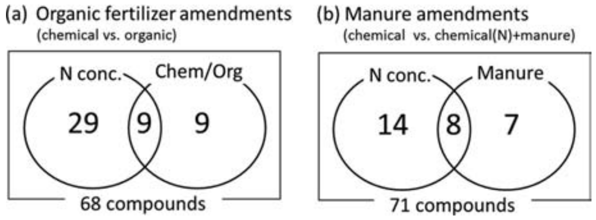

Figure 3. Venn diagrams of the metabolites for which levels were significantly altered by either or both N concentration in shoots and treatments: (a) "Chem/Org" indicates the difference between chemical and organic treatments; (b) "Manure" indicates the difference between chemical and chemical (N) plus manure treatments. The number of total compounds in each Venn diagram includes that of compounds detected and showed no interaction.

To determine the effects of manure amendments on metabolites, ANCOVA was performed with the 71 peak areas using the N concentration and the C and C+M treatments as predictor variables, except for the C-L0 and O treatments. The interaction of the N concentration and manure was not significant for all compounds. Seven compounds showed significant differences for manure amendment alone, eight compounds were significantly affected by both manure amendment and N concentration, whereas 14 compounds showed significant responses to the N concentration alone (Figure 3). Groups of similar metabolites, such as organic acids and sugars, were similarly affected by manure amendment. Under C+M treatment, five organic acids (malic, succinic, ferulic, ascorbic, and isoascorbic acids) and three sugars/sugar phosphate (glucose, glucose-6-phosphate, and xylose) compounds showed significantly lower concentrations, whereas four sugars (arabinose, ribose, *myo*-inositol, and glycerol) exhibited higher concentrations compared with the C treatment.

■ DISCUSSION

Validity of the Samples Obtained. Because growth and nitrogen nutrition greatly affect metabolite profiles, we compared the sizes and nutritional levels of the samples. In this study, we set the N input level of the C+M treatment to the same values as the lower levels of C treatments, as the N concentration was expected to with manure amendments, and we successfully obtained samples from the C+M treatment with N concentrations (17.4–20.1 g kg^{-1} DW) within the N range (11.8–23.1 g kg^{-1} DW) of the C treatment (Table 2). The obtained samples showed no deficiency or stress symptoms, except for the C-L0 treatment, which resulted in an N deficiency.

Comprehensive View of Metabolic Alterations. PCA revealed that the organic fertilizer and manure amendments differently affected the composition of the metabolites detected using GC-MS, although the plant N content was the most influential factor. PC1 contributed 33.4% of the total variance and was significantly associated with the plant N concentration ($P < 0.01$). Two previous field experiments involving the split-plot design of applied inorganic N (chemical fertilizer) and manure rates to evaluate the effects of manure amendments on metabolite composition in the leaves and roots of the radish[20] and leaves of komatsuna (*B. rapa* var. Perviridis),[34] using the same GC-MS-based methodology, generated PC1 scores with strong positive correlations to the amount of plant N absorbed and not the N concentration. This difference might reflect lower N concentrations in the C+M treatment compared with those in the C treatment, even if the treatments showed similar fresh weights (i.e., C-L2 and C+Md) (Table 2). Consistent with the PCA results of previous studies, most amino acids showed high PC1 loading scores, reflecting a close association with the plant N status. PC2 contributed 15.6% of the total variance and discriminated between the O and C treatments. This result is the first observation using PCA on metabolic alterations corresponding to the amendment of fast release organic fertilizers; much smaller differences were detected in a similar experiment using komatsuna.[34] This difference between komatsuna and mizuna, which are both *B. rapa*, might reflect the different responses to inorganic N. Kondo et al. reported that the influence of the N fertilizer application rate on NO_3^- concentration in mizuna differed from that of komatsuna[21] and suggested that the NO_3^- concentration of mizuna could abruptly increase with increasing N fertilizer application rate compared with komatsuna. PC4 contributed only 6.6% of the total variance, but clearly discriminated between the C+M and C treatments. These results indicate that manure amendments certainly affect the composition of metabolites in mizuna.

We characterized the effects of fertilizer amendments using a variety of organic fertilizers and manure. Three types of organic fertilizers (fish cake, chicken droppings, and rapeseed cake) were used to determine the effects of the origin of the fertilizers. Two types of manure processing (fresh and dried) amended to determine the effects of the biological function of manure. We expected that different types of organic fertilizers and the preparation of manure would alter the PCA scores of compounds in mizuna. Surprisingly, the types of organic fertilizers and preparation of manure did not affect the principal components.

On the basis of the results of the PCA, ANCOVA showed significant alterations of metabolites associated with (a) organic fertilizer amendment (fish cake, chicken droppings, and rapeseed cake) and (b) manure, separately from the effects of plant N concentration (Figure 3). The plant N concentration was the major factor affecting the amount of many compounds, rather than the direct effect of fast-release organic fertilizers or manure. There was a large difference in the number of compounds significantly altered due to the amendments between fast-release organic fertilizers and manure. Only two compounds were significantly and similarly altered through organic fertilizer and chemical (N) plus manure treatments (increase of phosphoric acid and decrease of spermidine), and other compounds showed different behaviors. This result indicated that organic fertilizers and manure had different influences on metabolites in mizuna. Because the effects of organic fertilizer amendment were evaluated by comparing organic and chemical fertilizer treatments in the present study, the influence of chemical fertilizers should be considered; the effect of manure amendment was evaluated by comparing the difference between chemical fertilizer treatment with and without manure to evaluate the effect of manure amendment.

Effects of Fast-Release Organic Fertilizer Amendments. This paper is the first to show clear metabolic alterations in plants through the amendment of fast-release organic fertilizer. The evaluation of organic matter amendment on crop quality and the influence of input systems of "organic" and "conventional" farming on major components (e.g., sugars, amino acids, and antioxidants) have been compared in many studies;[5,22−24] however, inconsistent results have made it impossible to reach any consensus.

A metabolomics approach generates results regarding global alterations of metabolites, which are potentially associated with the major components discussed above. Using metabolite profiling, Zorb et al. reported a significant decrease of 14 and 11 amino acids in the ears and grains of field-grown wheat under organic compared with conventional farming systems.[16] The plant N content strongly influences the levels of amino acids.[18,25] Our experiment set several N input levels to respective treatments and focused on the differences between the organic and chemical fertilizers applied to distinguish the factors associated with organic fertilizer and plant N content. We also observed significant alterations of 18 compounds (including the reduction of 5 amino acids) with the application of organic fertilizer compared with chemical fertilizer. Some differences in the pattern of N supply (e.g., inorganic form or dose) between organic and chemical fertilizers from sowing until harvesting were not reflected in the plant N content at harvesting and might have induced significant alterations of the amino acids in mizuna. It has been proposed that throughout the cultivation period, the N supply patterns are derived from differences between organic and chemical fertilizers and are reflected in the composition of metabolites in the crops at harvest. To confirm this idea, the temporal monitoring of the alteration of crop metabolites and N contents under artificially varied N supply patterns or changing rates of organic and chemical fertilizers should be conducted, with reference to studies of temporal metabolic monitoring.[26,27]

Relatively minor metabolites were significantly increased in response to fast-release organic fertilizer amendment, for example, tryptophan, *myo*-inositol-2-phosphate, fumaric acid, and maleic acid, compared with significant increases of major amino acids after chemical fertilizer application. The diversity of metabolic alterations in crops with organic fertilizer amendment should also be quantitatively evaluated using appropriate statistical methods.

The results of the present study showed that organic fertilizer amendments influenced the composition of metabolites in mizuna. Thus, mizuna might be suitable for the evaluation of the effects of organic fertilizer amendment on metabolites, reflecting a relatively sensitive response to applied materials.

Effects of Manure Amendments. This paper is the first to demonstrate clear metabolic alterations in mizuna with manure application, although obvious metabolic responses to the manure application rate were previously shown in field-grown radish and komatsuna compared with that of chemical fertilizer.[20]

The relative concentrations of five sugars and three organic acids were altered due to manure application in a similar manner as demonstrated in one or both of the previous studies on Brassicaceae mentioned above (Table 3). These consistent behaviors reflect compositional changes in sugars and decreases in organic acids.

The metabolism of sugars is influenced through light intensity and nutrient regimen, particularly the carbon/N balance.[28,29] Contrary to the reduction of most whole sugars at high N levels observed in the metabolite profiling of spinach,[18]

Table 3. Similar Alteration of Metabolites Corresponded to Manure Application in Brassicaceae[a]

metabolite	radish (*R. sativus* L.)	komatsuna (*B. rapa* var. Pervidis)	mizuna (*B. rapa* L. var. Nipposinica)
sugars			
arabinose	+		+
ribose	+		+
myo-inositol		+	+
glucose	−		−
xylose	−	−	−
oragnic acids			
malic acid	−		−
succinic acid	−	−	−
ferulic acid		−	−

[a] Plus (+) and minus (−) indicate significantly increased and decreased with manure application compared to without manure, respectively. Data of radish (leaves and roots) were from Okazaki et al.[20] Data of komatsuna (leaves) were from Okazaki et al.[34].

several compounds, such as arabinose, were increased, whereas others, such as xylose, were reduced with manure application, irrespective of the influence of N level. Arabinose, which is an aldopentose, partially exists as an arabinan through α-1,5 interactions between two arabinoses (or the constituents of arabinoxylan) with xylose in plants. Arabinans have been identified in many cell walls and are typically considered as part of the pectic network.[30] Furthermore, Jones et al. showed that cell wall arabinans are essential for the proper functioning of stomatal guard cell walls and proposed a role for arabinan in the maintenance of fluidity in the pectic network.[31] Xylose, which is also an aldopentose, is a constituent of various cell wall matrix polysaccharides classified as hemicelluloses, for example, xylan, glucuronoarabinoxylan, and xyloglucan. Gomez et al. demonstrated that the concentrations of arabinose and xylose in the 2-week-old leaves of *Arabidopsis thaliana* comprised approximately 15 mol % noncellulosic sugar, although the level of arabinose in the leaves was far lower than that in the embryos and germinating seeds.[32] From these observations and the results of our previous studies (Table 3), the application of manure could potentially improve the quality parameters associated with cell walls, such as food texture, through changes in the sugar concentrations.

Furthermore, the primary metabolites detected using GC-MS exhibit a close association with other secondary metabolites;[33] thus, it is worth investigating whether there is a relationship between secondary and primary metabolites for improving the quality of crops.

Concluding Remarks. A total of 71 compounds were detected in the shoots of pot-grown mizuna using GC-MS-based metabolite profiling. PCA showed contributions of 33.4, 15.6, and 6.6% to the total variance from plant N concentration, fast-release organic fertilizer application, and manure application, respectively. The evaluation of the effects of organic matter amendment was focused on N and revealed that the N input levels, the amendment of fast-release organic fertilizers, and manure amendments also changed the composition of metabolites in mizuna. Surprisingly, the variety of organic fertilizers (varieties of origin) and manure (dried or fresh) did not affect the PCA scores. Under organic fertilizer amendment, 18 compounds were significantly altered compared with the chemical fertilizer amendment. In particular, several compounds (e.g., glutamine, asparagine, tryptophan, and *myo*-inositol-2-phosphate) were clearly altered in response to organic or chemical fertilizer amendments. However, under manure amendment, 15 compounds were significantly altered compared to cultivation without manure amendment, reflecting changes in the composition of sugars and the reduction of some organic acids. These observations will be useful to further characterize the effects of organic matter amendments on crop metabolites.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional figure and tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

This study was partly supported by a grant of the Ministry of Education, Culture, Sports, Science and Technology in Japan.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Yukie Matsuyama for technical assistance in operating the GC-MS.

■ ABBREVIATIONS USED

GC, gas chromatography; MS, mass spectrometry; N, nitrogen; P, phosphorus; K, potassium; TCA, tricarboxylic acid; PCA, principal component analysis; PC, principal component; ANCOVA, analysis of covariance

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