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A Highly Sensitive, Quick and Simple Quantification Method for Nicotianamine and 2′-Deoxymugineic Acid from Minimum Samples Using LC/ESI-TOF-MS Achieves Functional Analysis of These Components in Plants

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Short Communication

A highly sensitive quantitative method for assaying nicotianamine (NA) and 2′-deoxymugineic acid (DMA) using liquid chromatography/electrospray ionization time-of-flight mass spectrometry (LC/ESI-TOF-MS) was developed. The amino and hydroxyl groups of NA and DMA were derivatized using 9-fluorenylmethoxycarbonyl chloride. The amounts of NA and DMA in 10 µl of xylem sap from rice cultivated under iron (Fe)-sufficient and Fe-deficient conditions were quantified without concentration. In Fe-sufficient plants, the concentrations of NA and DMA were almost equal to that of Fe. In Fe-deficient plants, the concentration of NA did not change significantly, whereas that of DMA increased markedly.

Keywords: 2′-Deoxymugineic acid • Fe deficiency • Nicotianamine • Xylem transport.

Abbreviations: BPDS, bathophenanthrolinedisulfonic acid; CE, capillary electrophoresis; DMA, 2′-deoxymugineic acid; FMOC-Cl, 9-fluorenyl methoxycarboxyl chloride; GC/MS, gas chromatography/mass spectrometry; ICP/MS, inductively coupled plasma/mass spectrometry; LC, liquid chromatography; LC/MS, liquid chromatography/mass spectrometry; LC/ESI-TOF-MS, liquid chromatography/electrospray ionization time-of-flight mass spectrometry; MAs, mugineic acid family phytosiderophores; MS, mass spectrometry; NA, nicotianamine; NL, *N*ε-nicotyl-lysine.

Iron (Fe) and zinc (Zn) micronutrient deficiencies are the most prevalent problems in human nutrition. Understanding the absorption and transport of metals in plants will aid in producing crops that accumulate high levels of Fe and Zn. Nicotianamine (NA) and 2′-deoxymugineic acid (DMA) are chelators of metals in plants and play important roles in the uptake and transport of metals (Takagi 1976).

DMA is indispensable when graminaceous plants take up Fe from the soil. Although Fe is abundant in soils, its availability to plants is sometimes low because it primarily forms highly insoluble ferric compounds under aerobic conditions. To overcome the generally low supply of Fe, higher plants have evolved special strategies to take up Fe (Römheld and Marschner 1986). Graminaceous plants use one of these strategies, called strategy II, and non-graminaceous plants use strategy I. In strategy II, graminaceous plants produce and secrete the mugineic acid family phytosiderophores (MAs), chelators of Fe(III), into the rhizosphere (Takagi 1976). DMA is the MA produced by rice. The secreted MAs chelate and solubilize rhizospheric Fe(III). The MA–Fe(III) complex is then absorbed by the root via the ZmYS1 transporter and the YS1-like (YSL) transporters HvYS1 and OsYSL15 (Curie et al. 2008).

NA, a biosynthetic precursor of DMA found in all higher plants, is converted to DMA via 3″-keto acid (**Supplementary Fig. S1**). Synthases responsible for these reactions (NA

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synthase, NA aminotransferase and DMA synthase) are induced under Fe-deficient conditions (Inoue et al. 2003, Bashir et al. 2006, Inoue et al. 2008). NA plays an important role in transporting metals in the plant body (Ling et al. 1999, Takahashi et al. 2003).

NA and DMA are important in the xylem transport of metals (Pich and Scholz 1996, Liao et al. 2000, Weber et al. 2002), as some NA– and MA–metal complexes have been detected in xylem sap. However, the manner in which these components and their transporters function cooperatively in metal transport is unclear. Unfortunately, such studies are hindered by the fact that HPLC-based quantification of NA and DMA requires large sample amounts, making the analysis of NA and DMA in rare samples (such as transgenic plants) especially difficult. Recently, NA–metal and MA–metal complexes have been detected using gas chromatography/mass spectrometry (GC/MS), inductively coupled plasma/MS (ICP/MS), liquid chromatography/MS (LC/MS), capillary electrophoresis/MS (CE/MS) and CE (Xuan et al. 2007), but the sensitivities of these assays are insufficient to quantify the low levels (millimolar) at which the complexes are present.

We previously reported a highly sensitive quantification method for NA (Wada et al. 2007). Our objective in this study was to refine this method to measure NA and DMA in small samples using one assay, with fewer sample preparation steps. Fluorenylmethoxycarbonylation of DMA and NA enabled highly sensitive quantification using LC/electrospray ionization (ESI)-time-of-flight (TOF)-MS. As a first step, xylem sap from rice cultivated under Fe-deficient and Fe-sufficient conditions was analyzed. Here, we report the first detection of the intermediate in the conversion of NA to DMA, 3″-keto acid, as well as NA and DMA.

To determine the optimal conditions for derivatization, we tested the reaction at several pH levels (7.0–11.0), reaction temperatures (25, 40, 50 and 60°C) and reaction times (5–60 min). At pH 8.0, the most intense signals were obtained for NA and DMA, with nicotyl lysine (NL; used as an internal standard for quantification) scoring the highest signal intensity (**Fig. 1A**). The reaction took longer to reach the maximum signal intensity at lower temperatures (data not shown). All reactions reached a plateau after 10 min (**Fig. 1B**), and the signal intensity of NL was highest at 5 min and gradually decreased after 10 min. Based on these results, all further analyses were carried out at pH 8.0 and 60°C, and were allowed to proceed for 15 min. Miyano et al. (1985) reported that metals in solution inhibited derivatization by 9-fluorenyl methoxycarboxyl chloride (FMOC-Cl), and

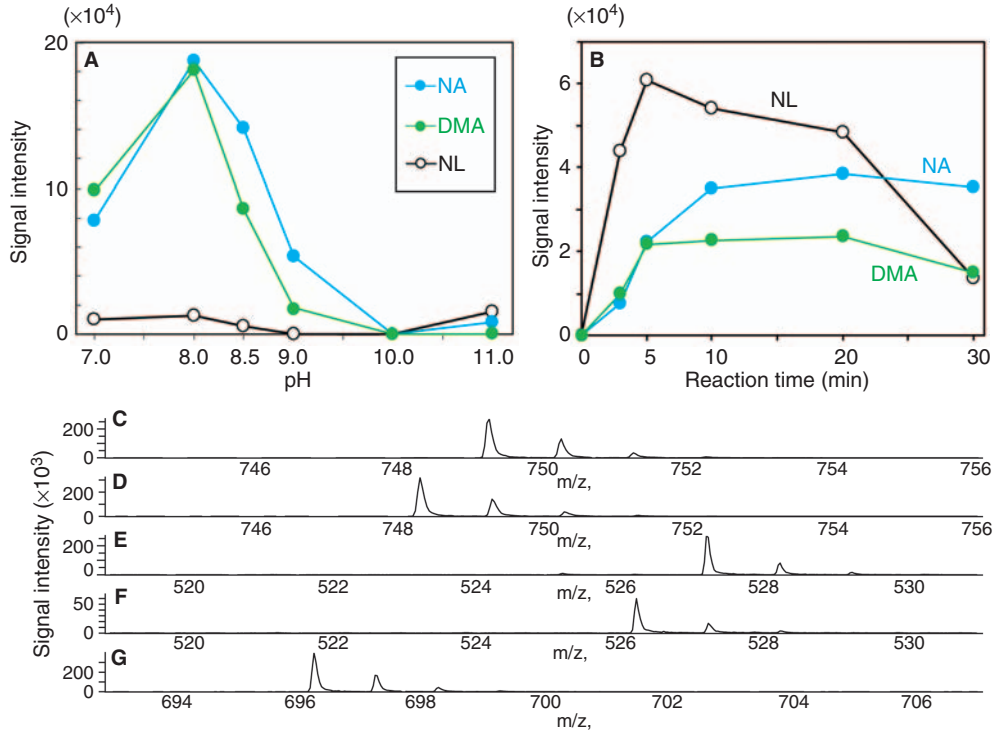

Fig. 1 Determination of derivatized NA, DMA and NL. (A) Derivatizing reactions at various pHs. NA, DMA and NL were derivatized under the conditions of 60°C, 10 min, and pH 7.0, 8.0, 8.5, 9.0, 10.0 or 11.0. (B) Derivatizations for various reaction times. NA, DMA and NL were derivatized under the conditions of 60°C, pH 8.0, and 0, 3, 5, 10, 20 or 30 min. (C–G) Detection of DMA, NA and NL by LC-ESI-TOF-MS. (C) $[DMA\text{-}FMOC_2+H]^+$ (*m/z*: 749); (D) $[NA\text{-}FMOC_2+H]^+$ (*m/z*: 748); (E) $[DMA\text{-}FMOC+H]^+$ (*m/z*: 527); (F) $[NA\text{-}FMOC+H]^+$ (*m/z*: 526), (G) $[NL\text{-}FMOC_2+H]^+$ (*m/z*: 696).

EDTA blocked this inhibition. Therefore, 10 µl of 50 mM EDTA was added to the reaction solution. When 10 µl of 50 µM NL, DMA or NA was reacted and measured using LC/ESI-TOF-MS, we detected $[DMA\text{-}FMOC_2+H]^+$ (*m/z*: 749), $[DMA\text{-}FMOC+H]^+$ (*m/z*: 527), $[NA\text{-}FMOC_2+H]^+$ (*m/z*: 748), $[NA\text{-}FMOC+H]^+$ (*m/z*: 526) and $[NL\text{-}FMOC_2+H]^+$ (*m/z*: 696) (**Fig. 1C–G**).

Quantification of DMA and NA was carried out by comparison and calculation of each peak area. The peak areas of $[DMA\text{-}FMOC_2+H]^+$ and $[NA\text{-}FMOC_2+H]^+$ were selected and both peak areas were divided by the peak area of $[NL\text{-}FMOC_2+H]^+$. These peak area ratios were used to calculate the quantities of NA and DMA. Calibration curves were generated for NA and DMA. For both NA and DMA, calibration curves from 10 to 3,000 fmol gave R^2 values of 0.999 (data not shown). The NA and DMA concentrations in xylem sap containing additional authentic samples were also calculated, and the detected amounts of NA and DMA coincided with the calculated values (**Fig. 2**).

To quantify NA and DMA at the same time, the reaction and LC elution parameters needed to be changed from the NA quantification method established by Wada et al. (2007). Under the LC conditions of Wada (2007), the derivatives of NA and DMA were eluted overlapping each other, making their quantification very difficult, because the difference between their molecular weights is only 1, and the intensity of the $^{13}C\text{-}[NA\text{-}FMOC_2+H]^+$ peak, which reaches approximately 30% of that of the $^{12}C\text{-}[NA\text{-}FMOC_2+H]^+$ peak, is sometimes large enough to interfere with the evaluation of the peak area of small amounts of $^{12}C\text{-}[DMA\text{-}FMOC_2+H]^+$. Instead of gradient elution from water to CH_3CN, elution with a solvent of 0.5% formic acid, 36% water and 63.5% CH_3CN improved the separation of these compounds by LC and, moreover, this solvent greatly reduced the elution time from 60 min to 8 min. Another problem with the previous derivatizing procedure was also solved. Lowering the pH of the reaction mixture from 8.0 to 4.0 after the FMOC derivatization kept $NL\text{-}FMOC_2$ unchanged for at least a few days, while it gradually degraded in the previous procedure in a rather short time, about after 1 h (data not shown).

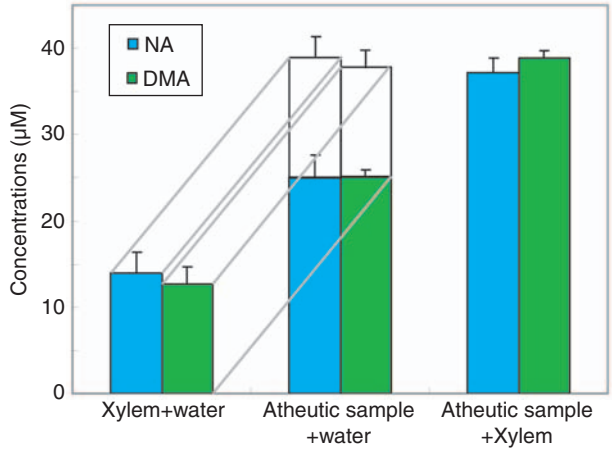

Fig. 2 Validation of the quantification method. The concentrations of NA and DMA in rice xylem sap, authentic NA and DMA samples, and xylem sap spiked with authentic NA and DMA were analyzed ($n = 3$; means ± SD).

Derivatization using FMOC-Cl enhances the hydrophobicity of compounds and enhances the efficiency of ionization by ESI, which increases the sensitivity of LC/ESI-TOF-MS. This method is approximately 1,000-fold more sensitive than HPLC, and NA and DMA could be quantified in samples as small as a rice seed embryo. Thus, metal storage in seeds and the mechanism of metal transport to plant tissues via NA or DMA can be investigated using this method. Furthermore, this reaction can be performed in an aqueous solution, whereas other derivatizations used for LC/ESI-TOF-MS, such as silanization, are commonly inhibited by water.

With this improvement, 3″-keto acid, an intermediate in the pathway from NA to DMA, was detected for the first time. This component had been thought to be easily collapsed. The derivatization in aqueous solution seems to work well for such compounds as 3″-keto acid which is unstable under the dry derivatizing condition. 3″-Keto acid, the intermediate compound in the conversion of NA to DMA, was detected for the first time after first being synthesized in vitro from NA. 3″-Keto acid was then detected as $[3''\text{-keto acid-FMOC}+H]^+$ (*m/z*: 525) (**Fig. 3A**). After the conversion of 3″-keto acid to DMA, only DMA peaks were observed as $[DMA\text{-}FMOC_2+H]^+$ (*m/z*: 749) (data not shown) and $[DMA\text{-}FMOC+H]^+$ (*m/z*: 527) (**Fig. 3B**).

We quantified the concentrations of NA and DMA in xylem sap from rice plants. To our knowledge, this is the first detection of NA in the xylem sap of graminaceous plants. In Fe-sufficient plants, the concentration of both NA and DMA was 9.6 µM (**Fig. 4A**). Furthermore, the total amount of Fe in rice xylem sap under Fe-sufficient conditions was 11.0 µM, close to the concentrations of NA and DMA (**Fig. 4B**). In addition, 75% of the Fe in xylem sap was found to be ferrous Fe (**Fig. 4B**), which was unexpected, as the most common form of Fe in xylem sap was previously thought to be ferric Fe (von Wirén et al. 1999). Half of this ferrous Fe changed to ferric Fe when the xylem sap samples were stored at room temperature for 3 h (data not shown).

Citric acid in non-graminaceous plants (López-Millán et al. 2000) and histidine in hyperaccumulators (Liao et al. 2000) have been suggested to be the main ligands for metals in xylem. The concentration of citric acid in rice xylem sap is about 150 µM (Yokosho et al. 2008), and citric acid mainly binds to ferric Fe. Previous studies to determine Fe ligands in xylem sap focused on those that bind ferric Fe, but in this study we found that Fe was also transported as a form of

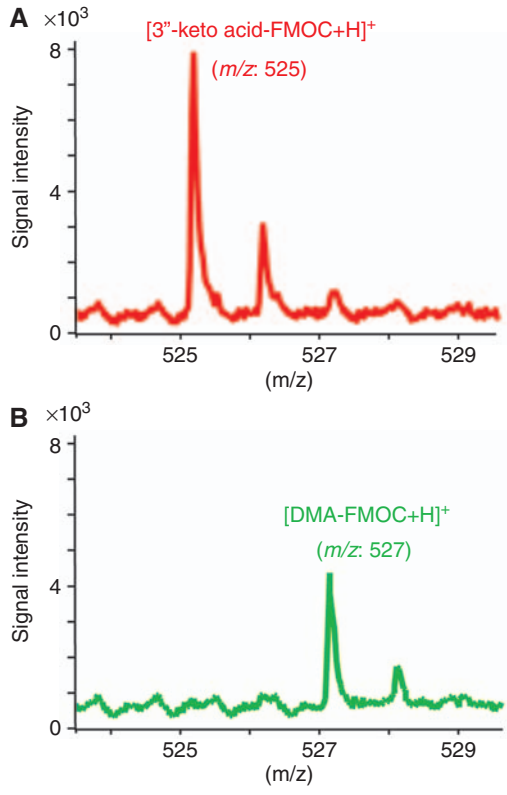

Fig. 3 Detection of 3″-keto acid. 3″-Keto acid was synthesized from NA, and DMA was synthesized from 3″-keto acid by HvNAAT and HvDMAS, respectively. The peak of the 3″-keto acid was detected after the conversion of NA by HvNAAT (A), but was not detected after the subsequent reaction catalyzed by HvDMAS (B).

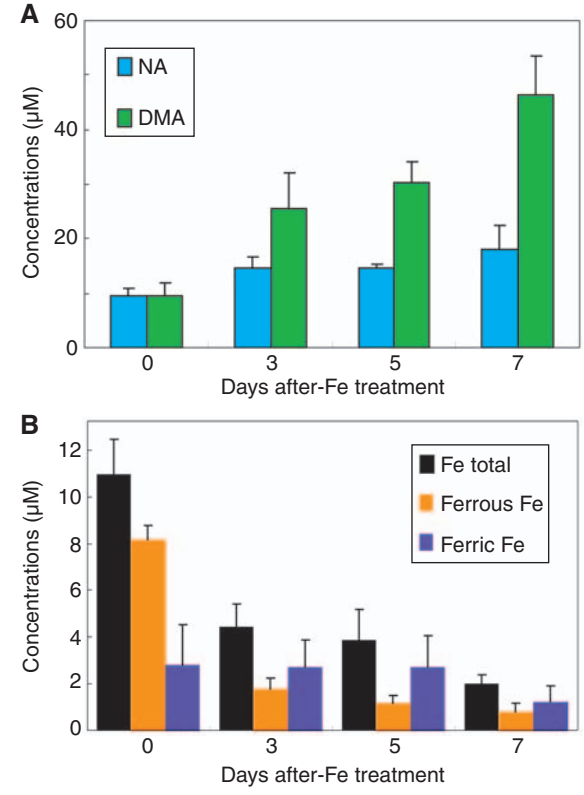

Fig. 4 Components in rice xylem sap. (A) Concentrations of NA and DMA in rice xylem sap after Fe deficiency treatment. Rice xylem sap from rice cultivated under Fe-sufficient and Fe-deficient conditions was collected, and the concentrations of NA and DMA were quantified ($n = 3$; means ± SD). (B) Concentrations of ferrous Fe and ferric Fe in xylem sap. Using the sample from (A), the concentrations of ferrous Fe and ferric Fe were quantified ($n = 3$; means ± SD).

ferrous Fe via the rice xylem. The pH of rice xylem sap was about 5.5, and at this low pH the speed of autoxidation may be slower than expected. We believe that citrate is still a strong candidate for the Fe ligand in rice xylem sap, but, because NA strongly binds ferrous Fe, it may also function as a ligand for ferrous Fe in rice xylem sap. Conversely, histidine is known as a minor amino acid in rice xylem sap (Fukumorita and Chino 1982). We suggest that NA and MAs also play an important role in the transport of Fe to the shoot because NA and MAs exist in xylem, and the expression of YSL family transporters is also observed in vascular tissues (Curie et al. 2008).

In Fe-deficient plants, the NA concentration did not change significantly over time, whereas that of DMA increased markedly (**Fig. 4A**). At day 7, the DMA concentration was approximately 5-fold that on day 0. At the same time, the concentration of total Fe decreased markedly from day 0 (11.0 μM) to day 3 (4.2 μM; **Fig. 4B**). The concentration of ferric Fe did not change from day 0 to day 5 (2.7 μM), but began to decrease from day 5 (2.7 μM) to day 7 (1.2 μM). This suggests that DMA may be able to solubilize apoplastic Fe deposited on the cell and/or xylem walls. Under Fe-deficient conditions, rice plants produce more DMA, increasing its concentration in xylem and remobilizing Fe from those compartments. At the same time, the concentration of NA in xylem sap was maintained at a specific level (10 μM). This may contribute to the distribution and homeostasis of other metals, such as Cu and Zn which bind to NA.

Materials and Methods

All reagents were of analytical grade. FMOC-Cl was purchased from Tokyo Kasei (Japan) and NL was synthesized following Wada et al. (2007).

NL was added to the sample at the first step of each derivatizing reaction. For derivatization, FMOC-Cl was used. First, 10 μl of 1 M sodium borate (pH 8.0) was added to 5 μl of sample solution to adjust the pH of the solution to 8.0. Then, 10 μl of 50 mM EDTA (pH 8.0) was added. A 40 μl

aliquot of 50 mM FMOC-Cl was then prepared by dissolving it in CH_3CN, and it was added to the solution. The reaction mixture was incubated at 60°C for 15 min. After the reaction, the pH of the sample solution was adjusted to 4.0 using 5% formic acid to stop the reaction. To obtain calibration curves, authentic samples of DMA (10–3,000 fmol), NA (10–3,000 fmol) and NL (100 fmol) were weighed and mixed before the reaction.

LC-ESI-TOF-MS measurement was carried out using a JSM-T100LC AccuTOF (JEOL, Tokyo, Japan) in ESI+mode. The desolvent temperature was 300°C, the orifice 1 temperature was 120°C and the ESI needle voltage was 2,000 V, except for **Fig. 1B** (2,500 V). The LC separation was performed using a Synergi Hydro RP column (4 m, 80 A, 150×2.00 mm; Phenomenex, Torrance, CA, USA) with a solvent of 0.5% formic acid, 36% water and 63.5% CH_3CN. Using this system, a single run could be completed within 8 min. The detection mass range (*m*/z) was set from 200 to 1,000.

3″-Keto acid was prepared by conversion of NA with recombinant HvNAAT (NA aminotransferase in barley), and DMA was likewise prepared from 3″-keto acid with recombinant HvDMAS (DMA synthase in barley) following the protocol of Bashir et al. (2006). HvNAAT and HvDMAS are biosynthetic enzymes involved in conversions from NA to 3″-keto acid and from 3″-keto acid to DMA (**Supplementary Fig. S1**).

Rice cv. Nipponbare was cultivated hydroponically for 3 weeks in an Fe-sufficient solution and then moved to an Fe-deficient solution following Higuchi et al. (2001). The plants were detopped at a height of 3 cm from the root. The surface of the excised leaf sheath was gently wiped, and a tube filled with cellulose was placed on the cut end. The entire length of the tube was covered with aluminum foil to prevent the DMA from decomposing due to light. Xylem sap was collected for 30 min from 2 h after sunrise. Xylem sap was collected after the rice plants were exposed to Fe deficiency for 0 (before the exposure), 3, 5 and 7 d. Twenty-four rice plants were prepared as donors for each day (a total of 96 plants). The xylem sap collected in cellulose was filtered with a 0.65 µm filtering column (Millipore). The extracted xylem sap was stored at −20°C until further analysis. Because the amount of xylem sap differed, 18 samples with median values of these xylem sap amounts were selected from the 24 samples, and six individuals each were pooled at random into three tubes for the measurement.

Fe in xylem sap was determined using bathophenanthrolinedisulfonic acid (BPDS) to detect BPDS-Fe^{2+} via UV absorption (533 nm) using a NanoDrop spectrophotometer (NanoDrop, Wilmington, DE, USA). First, 5 µl of 0.1 M BPDS was added to 10 µl of xylem sap and analyzed for the concentration of ferrous Fe. Standard curves were generated using Fe(III)EDTA reduced by ascorbate. After the Fe^{2+} in the xylem sap was measured, 5 µl of 1 M ascorbate was added and incubated overnight in darkness. The absorption of the incubated mixture was measured as the total amount of Fe. The amount of ferric Fe was calculated as the difference between the total Fe and Fe^{2+} contents.

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