Title

Development of a highly sensitive, quick, and easy LC-ESI-TOF-MS method to quantify nicotianamine and 2'-deoxymugineic acid in plants

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INTRODUCTION

The study of metallic nutrients in plants is important for both agriculture and human health. Fe and Zn micro-nutrient 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. NA and the mugineic acid family phytosiderophores (MAs) are chelators of metals in plants and play important roles in the uptake and transport of metals. DMA is the only MA produced by rice. 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. This mechanism may also function in the uptake of other metals, because MAs also strongly chelate Fe^{2+}, Ca^{2+}, Mn^{2+}, Zn^{2+}, Ni^{2+}, and Cu^{2+}. NA and DMA are also important in the transport of metals, as the leaves of plants that lack NA and/or DMA are chlorotic. NA– and MA–metal complexes may also be involved in long-distance transport, although the manner in which these components and their transporters function cooperatively in metal transport requires additional studies. However, such studies are hindered by the fact that HPLC-based quantification of NA and DMA requires relatively large sample amounts, making the analysis of NA and DMA in rare samples (*e.g.*, transgenic plants) especially difficult. 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 at the same time from small samples, with fewer sample preparation steps. The fluorenylmethoxycarbonylation of DMA and NA enabled highly sensitive quantification using LC-ESI-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.

MATERIALS AND METHODS

Derivatizing reaction. NA and DMA were derivatized using 9-fluorenyl methoxycarboxyl chloride (FMOC-Cl) to facilitate separation on a C18 column. 10 μl of samples were directly derivatized adding 10 μl of 1 M borate buffer, 10 μl of 50 mM EDTA, and 40 μl of 50 mM FMOC-Cl. Reactions for quantification analyses were carried out at pH 8.0 and 60°C, and were allowed to proceed for 15 min. To stop reaction, formic acid was added and adjusted pH to 4.0.

LC-MS condition. HPLC was carried out by Agilent 1100 series system. Column used was C-18 reversed phase column, Synergi Hydro RP column (4 m, 80 A, 150 × 2.0 mm; Phenomenex, Torrance, California, USA). Elution solution was mixture of acetonitrile, water, and formic acid, with ratio of 63.5 : 36 : 0.5. A JSM-T100LC Accu-TOF (JEOL, Tokyo) was used for MS detection in ESI+ mode with desolvent temperature 300°C, ESI needle voltage 2,000 V, orifice voltage 80 V, and ring guide voltage 2,500 V. MS signal from 200 to 1,000 was collected.

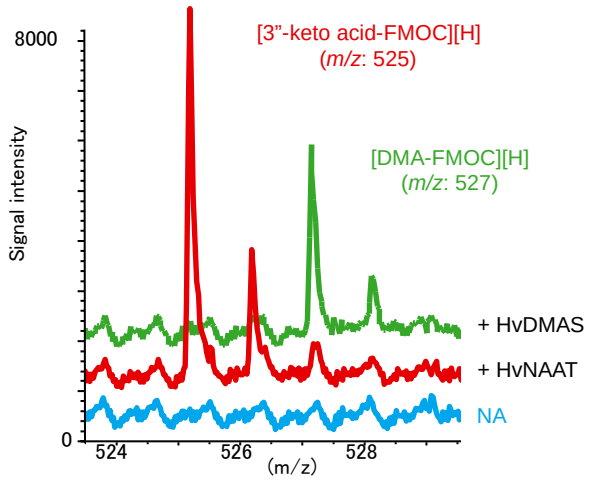

Figure 1. Detection of 3"-keto acid. 3"-keto acid was synthesized from NA, and DMA was synthesized from 3"-keto acid by HvNAAT and HvDMAS, respectively. The peak of 3"-keto acid was detected after the conversion of NA by HvNAAT, but it does not detected after the following reaction by HvDMAS.

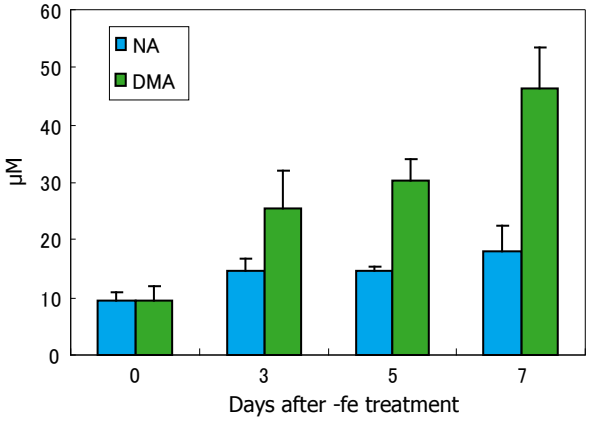

Figure 2. Concentrations of NA and DMA in rice xylem sap after Fe-deficient treatment. Rice xylem sap from rice cultivated under Fe-sufficient and Fe-deficient conditions was collected, and concentrations of NA and DMA were quantified. (n=3, means +SD)

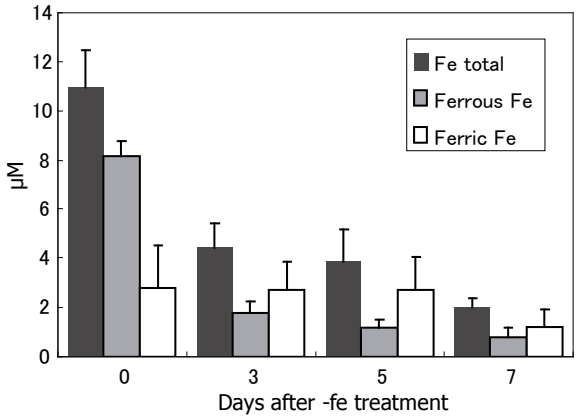

Figure 3. Concentrations of ferrous Fe and ferric Fe in xylem sap. Using the same sample of Figure 2, concentrations of ferrous Fe and ferric Fe were quantified. (n=3, means +SD)

Fe quantification. Ferrous Fe was determined using bathophenanthrolinedisulfonic acid (BPDS) to detect BPDS-Fe^{2+} via UV absorption (533 nm) using a NanoDrop spectrophotometer (NanoDrop, Wilmington, DE, USA). Total Fe content was quantified after adding ascorbate to samples. Concentration of ferric Fe was calculated as a difference between total Fe and ferrous Fe.

RESULTS

Establishment of a quantification method for NA and DMA. To determine the optimal conditions for derivatization, we tested the reaction at several pH levels (7.0 to 11.0), reaction temperatures (25, 40, 50, and 60°C), and reaction times (5 to 60 min). At pH 8.0, MS signals originated from NA and DMA, with NL (Nicotyl lysine, used as an internal standard for quantification) scoring the highest signal intensity. Regarding temperature, the reaction took longer to reach maximum signal intensity at lower temperatures. Regarding reaction time, all reactions reached a plateau after 10 min, and the signal intensity of NL was highest at 5 min and gradually decreased after 10 min. When 10 μl of 50 μM NL, DMA, or NA were reacted and measured using LC-ESI-TOF-MS, we detected [DMA-$FMOC_2$] + [H] (*m/z*: 749), [DMA-FMOC] + [H] (*m/z*: 527), [NA-$FMOC_2$] + [H] (*m/z*: 748), [NA-FMOC] + [H] (*m/z*: 526), and [NL- $FMOC_2$] + [H] (*m/z*: 696).

Detection of 3"-keto acid, a precursor of DMA. Most compounds containing amino and/or hydroxyl group can be detected in one run with our method, as FMOC binds to all amino acids and many other components with

amino or hydroxyl groups, in addition to NA and DMA. In addition, 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″-keto acid-FMOC] + [H] (*m/z*: 525) (Fig. 1). After the conversion of 3″-keto acid to DMA, only DMA peaks were detected as [DMA-$FMOC_2$] + [H] (*m/z*: 749), and [DMA- FMOC] + [H] (*m/z*: 527) (Fig. 1).

Quantification of NA and DMA in rice xylem sap. We quantified the concentrations of NA and DMA in xylem sap from Fe-deficient rice plants. In Fe-deficient plants, the concentration of NA did not change significantly over time, whereas that of DMA increased markedly (Fig. 2). At day 7, the concentration of DMA was approximately 5-fold that on day 0.

Quantification of Fe in rice xylem sap. The concentration of Fe in xylem sap was also determined. In Fe-sufficient plants, the total amount of Fe in rice xylem sap was 11.0 μM, with about 75% ferrous Fe. In Fe-deficient plants, the concentration of total Fe decreased markedly from days 0 (8.2 μM) to 3 (1.8 μM; Fig. 3). The concentration of ferric Fe did not change from days 0 to 5 (2.7 μM), but began to decrease from days 5 (2.7 μM) to 7 (1.2 μM).

Discussion

This method was approximately 1000-fold more sensitive than HPLC, and a sample as small as a rice seed embryo was sufficient to quantify NA and DMA. Thus, metal storage in seeds and the mechanism of metal transport to plant tissues via NA or DMA can be elucidated using this method. Furthermore, this reaction could be performed in an aqueous solution, whereas other derivatizations for LC-ESI-TOF-MS, such as acylation or silanization, are commonly inhibited by water. In Fe-sufficient plants, 75% of Fe in xylem sap was ferrous Fe. This was unexpected, as the most common form of Fe in xylem sap was previously thought to be ferric Fe. Free ferrous Fe is more harmful to plants, because it produces reactive oxygen species. Previous studies to determine Fe ligands in xylem sap have focused on those that bind ferric Fe. Because it binds strongly to ferrous Fe, NA may function as a ligand for ferrous Fe in xylem sap. The total amount of Fe in rice xylem sap under Fe-sufficient conditions was 11.0 μM, which was close to the concentrations of NA (9.6 μM) and DMA (9.6 μM). In Fe-deficient plants, the concentration of ferrous Fe in xylem sap decreased rapidly, whereas the concentration of ferric Fe remained constant until day 5. At the same time, the DMA concentration in the xylem sap increased markedly, suggesting that DMA may be able to solubilize apoplastic Fe deposited on cell and/or xylem walls. In the Fe-deficient condition, rice plants produced more DMA, increasing the concentration of DMA in xylem and re-mobilizing Fe in this compartment.

References

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