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Two Related Biosynthetic Pathways of Mugineic Acids in Gramineous Plants

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The biosynthesis of mugineic acids was studied by feeding ^2H- or ^{13}C-labeled compounds to water-cultured roots in several gramineous plants. The fate of labeled compounds was monitored by using ^2H- and ^{13}C-nuclear magnetic resonance. On investigating the proton changes during biosynthesis by feeding D,L-[3,3,4,4-d_4]-methionine (98.6% ^2H), ^2H-labeled 2′-deoxymugineic, mugineic, and 3-epihydroxymugineic acids were isolated from root washings of wheat (*Triticum aestivum* L. cv Minori), barley (*Hordeum vulgare* L. cv Minorimugi), and beer barley (*Hordeum vulgare* L. cv AM Nijo Tochigi), respectively. The ^2H-nuclear magnetic resonance study indicated that 12 deuteriums were incorporated into the labeled 2′-deoxymugineic acid, suggesting that three molecules of L-[3,3,4,4-d_4]methionine were combined. In comparison, one of the deuteriums at C-2′ position in the mugineic acid, and one each of the deuteriums at C-2′ and C-3 positions in the 3-epihydroxymugineic acid, were lost. However, all other deuteriums were incorporated in a manner similar to that of the labeled 2′-deoxymugineic acid. When [1,4′,4″-$^{13}C_3$]2′-deoxymugineic acid (20% ^{13}C) was fed to oat roots (*Avena sativa* L. cv Amuri II), avenic acid A, which was ^{13}C enriched at the corresponding positions, was obtained. These results revealed that L-methionine was the precursor for all these mugineic acids and that cleavage of the azetidine ring or hydroxylation of the 2′-deoxymugineic acid produced two related biosynthetic pathways in different gramineous plant species: L-methionine → 2′-deoxymugineic acid → avenic acid A in oat; and L-methionine → 2′-deoxymugineic acid → mugineic acid → 3-epihydroxymugineic acid in barley and beer barley.

Low yields due to iron chlorosis is a worldwide agricultural concern that is especially acute in calcareous soils. Foliar application of iron inorganic salts and soil treatment with synthetic chelates are currently accepted practices. However, because iron is not transported from old to new leaves, repeated treatments are required, making this approach costly.

Selecting and breeding plants resistant to iron deficiency may be a more economical approach. It is not uncommon to see both chlorotic and green plants growing in the same soil, so it is helpful to understand the mechanisms of iron acquisition in these green plants. According to Römheld and Marschner (1986), at least two strategies exist for acquiring iron in higher plants, based on their root responses to iron deficiency. Strategy I, often employed by dicotyledonous and nongrass monocotyledonous species, is characterized by an increase in the reducing capacity of roots, increased net excretion of protons, and enhanced release of reductants. In contrast, strategy II is characterized by the secretion of ferric chelating substances, which have been generically termed "mugineic acids." To date, only gramineous species have been found to employ this strategy (Römheld and Marschner, 1986; Nomoto et al., 1987). From the ecological point of view, strategy II has some advantages for adaptation to calcareous soils over strategy I because the former is able to solubilize sparingly soluble-inorganic iron and is less sensitive to high pH, elevated bicarbonate concentrations, and excessive Ca^{2+} and Mg^{2+} concentrations that are common features of calcareous soils (Römheld and Marschner, 1986). Thus, it may be best to breed plants that respond to iron deficiency by secreting mugineic acids.

For this purpose, an understanding of the biosynthesis of mugineic acids is a prerequisite because neither purification of related enzymes nor subsequent cloning of genes encoding synthetic enzymes of mugineic acids can be performed without elucidating the biosynthetic pathway. Since the chemical structure of mugineic acid isolated from barley plants was first determined (Takemoto et al., 1978), its biosynthesis has been explored. On feeding four ^{14}C-labeled amino acids and L-[1-^{13}C]Met to iron-deficient barley (*Hordeum vulgare* L. cv Minorimugi) roots, L-Met has been found to be the most efficient precursor for mugineic acid and 2′-deoxymugineic acid (Kawai et al., 1988). Similar in vitro findings by Shojima et al. (1989, 1990) have been documented. However, experimental data on the biosynthesis of other mugineic acids such as avenic acid A and 3-epihydroxymugineic acid, and on their relationship in the biosynthetic pathway, have yet to be published, although speculation has previously been attempted (Mori and Nishizawa, 1987; Kawai et al., 1988; Shojima et al., 1989, 1990).

In the present study, four mugineic acids were labeled by feeding either ^2H-labeled Met or ^{13}C-labeled 2′-deoxymugineic acid to water-cultured roots of four different species that were subsequently isolated from these plants. From the results of ^2H- and ^{13}C-NMR studies, two related biosynthetic pathways were postulated.

MATERIALS AND METHODS

Seeds of wheat (*Triticum aestivum* L. cv Minori), oat (*Avena sativa* L. cv Amuri II), barley (*Hordeum vulgare* L. cv Minorimugi), and beer barley (*Hordeum vulgare* L. cv AM Nijo Tochigi) were soaked overnight in water before sowing on vermiculite in a greenhouse. One week later, roots were carefully rinsed free of vermiculite, and about 80 selected seedlings of each species were then transplanted to continu-

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ously aerated nutrient solution in 3-m^3 plastic pots in an environmental chamber. Three-tenth strength Hoagland solution was supplied to the plants and contained 1.5 mM KNO_3, 1.5 mM $Ca(NO_3)_2$, 0.6 mM $MgSO_4$, and 0.3 mM $NH_4H_2PO_3$. Micronutrients were adjusted to full-strength Hoagland solution. The solution was adjusted to pH 6.0 with 0.1 N KOH and renewed every 2 d. Light intensity was about 40 W/m^2, and the light/dark periods and temperatures were 14/10 h and 17/10°C, respectively.

After a 2-week period, the plants were transferred to iron-free nutrient solution. When chlorosis developed, 200 μM D,L-[3,3,4,4-d_4]Met (98.6% ^2H, Merck Sharp & Dohme/Isotopes) (actually, only half this concentration; only the L-form would be used by the plants) was fed to the roots of wheat, barley, beer barley, and oats after the secretion of mugineic acids. To stimulate the Met uptake, surface-active agent Decaglyn 1-L was added at 40 $\mu g\ mL^{-1}\ pot^{-1}$. Ten micromolar [1,4′,4″-$^{13}C_3$]2′-deoxymugineic acid (20% ^{13}C), obtained by purification of root washings from wheat fed with 150 μM L-[1-^{13}C]-Met (99.0% ^{13}C, Isotec, Inc., Miamisburg, OH), was fed to the oat roots in a ferric complex form.

To isolate various mugineic acids, root washings were collected the following day by soaking the roots in distilled water from 3 to 5 h after the onset of the light period. The distilled water used for soaking was replaced every hour during the collection to maximize the yield. The root washings were then charged on Amberlite IR-120 (H^+) and the cationic fractions of 2 N NH_4OH were eluted. Subsequent chromatography on Dowex 50 WX8 was performed, and gradient eluates with ammonia-formate buffer (pH 2.5–3.1) provided a concentrated active fraction. Chelating activities were determined by the *o*-phenanthroline method (Takagi, 1976), and the mugineic acids in different fractions were separated by HPLC (Kawai et al., 1987). Gel filtration through Sephadex G-10 (H_2O as a solvent) for concentrated fractions of 2′-deoxymugineic acid, mugineic acid, and 3-epihydroxymugineic acid was finally conducted. Fractions of avenic acid A were charged on an AG 50 WX8 column and eluted subsequently by 2 N NH_4OH.

^1H-NMR spectra of isolated mugineic acids (in D_2O) were recorded on a 400-MHz spectrometer (JNM-EX400), and assignments of ^1H were accomplished by ^1H-^1H correlation

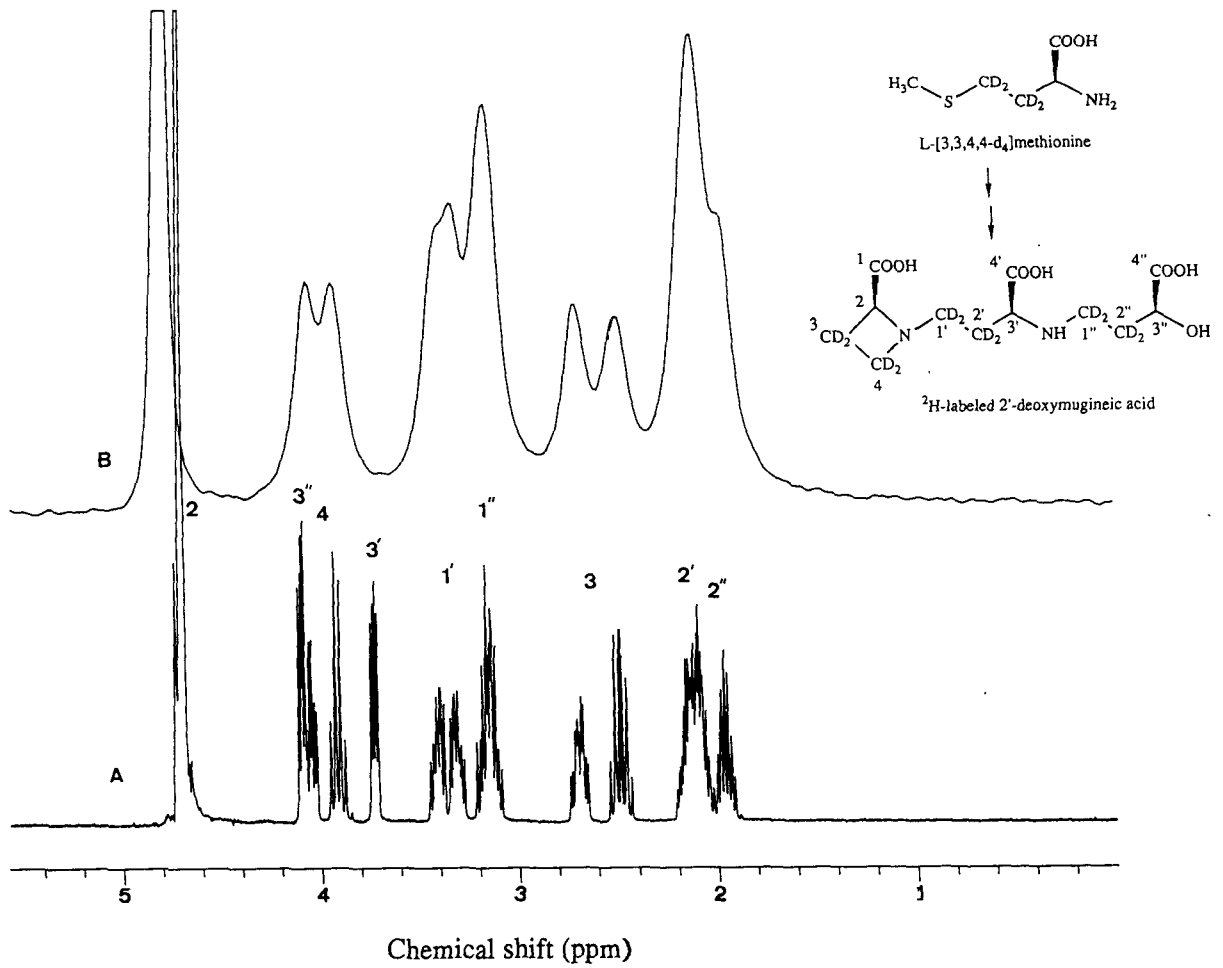

Figure 1. ^1H-NMR spectrum of unlabeled 2′-deoxymugineic acid (A) and ^2H-NMR spectrum of 2′-deoxymugineic acid biosynthesized from L-[3,3,4,4-d_4]Met (B) in wheat. Spectra were measured with 400-MHz (^1H-NMR, D_2O) and 61.3-MHz spectrometers (^2H-NMR, H_2O). The peak at 4.78 ppm represents the signal of either H_2O or D_2O.

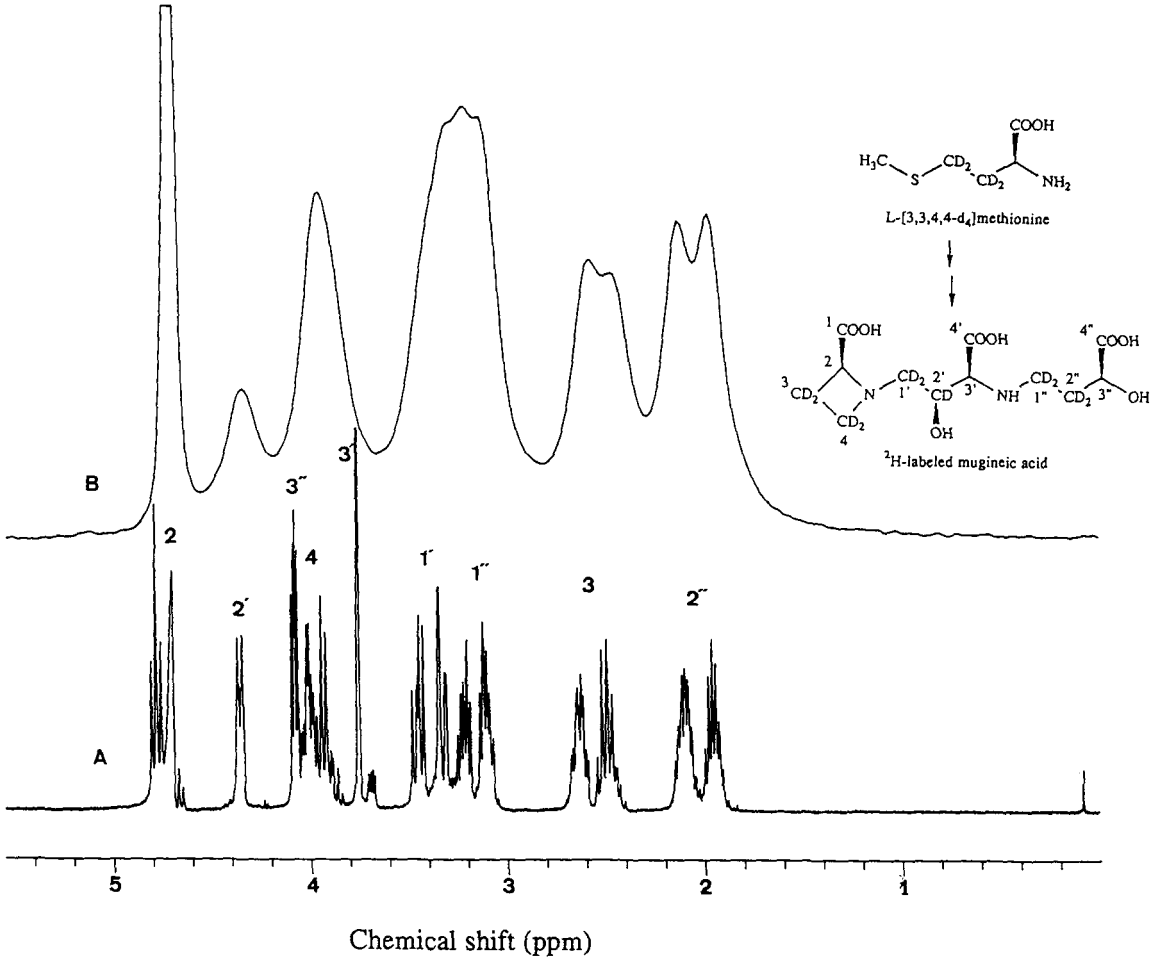

Figure 2. ^{1}H-NMR spectrum of unlabeled mugineic acid (A) and ^{2}H-NMR spectrum of mugineic acid biosynthesized from L-[3,3,4,4-d_4]Met (B) in barley. Spectral measurements are similar to those in Figure 1. The peak at 4.70 ppm represents the signal of either H_2O or D_2O.

spectroscopy, ^{1}H-detected multiple-bond heteronuclear multiple-quantum coherence, and ^{1}H-detected heteronuclear multiple-quantum coherence experiments (Iwashita, 1990).

The ^{2}H-NMR spectra (H_2O) were measured at 61.3 MHz (JNM-EX400 spectrometer) (Grant et al., 1982). Spectra of 10.0-μs pulse width requiring about 4000 scans were acquired at 2-s intervals with data acquisition (1.28 s) of 4096 points via a nondecoupling experimental mode. The natural abundance of ^{2}H in H_2O was used as a chemical shift reference to the ^{2}H experiment. The assignment was accomplished after analysis of the corresponding proton spectra.

^{13}C-NMR measurements of isolated avenic acid A (in 0.4 N ND_4OD) were performed with a 75.5-MHz spectrometer (GE-300) (Stothers, 1972). The spectra were obtained using 1-W broadband proton decoupling, 4.0-μs pulse width, 5.0-s delay time, 32,768 data points, and a spectral width of 20,000 Hz. About 10,240 scans were accumulated. Spectra were recorded using 2-Hz line broadening. 3-(Trimethylsilyl)propionic acid sodium salt-d_4 was used as an external reference for calibration of the chemical shift, and assignments for ^{13}C were accomplished by ^{13}C-^{1}H correlation spectroscopy. Calculation of ^{13}C enrichment was based on the line intensity of the C-3 position.

RESULTS AND DISCUSSION

Following the discovery of mugineic acid (Takemoto et al., 1978), several analogs such as hydroxymugineic acid (Nomoto et al., 1979), avenic acid A (Fushiya et al., 1980), and 3-epihydroxymugineic acid (Iwashita et al., 1981) have been located in different gramineous species. Except for L-Met, which is the precursor of 2′-deoxymugineic acid and mugineic acid in barley plants (Mori and Nishizawa, 1987; Kawai et al., 1988; Shojima et al., 1989; Shojima et al., 1990), information on the biosynthesis of mugineic acid analogs is as yet limited. In the present study, biosynthesis routes of different mugineic acids in four species were compared. Both ^{2}H- and ^{13}C-NMR were used for monitoring the fates of labeled compounds. Because ^{2}H chemical shifts correspond to ^{1}H shifts with the exception of a small isotope effect and broadening, we can speculate the assignment of ^{2}H-NMR peaks based on those of ^{1}H-NMR. Thus, proton changes

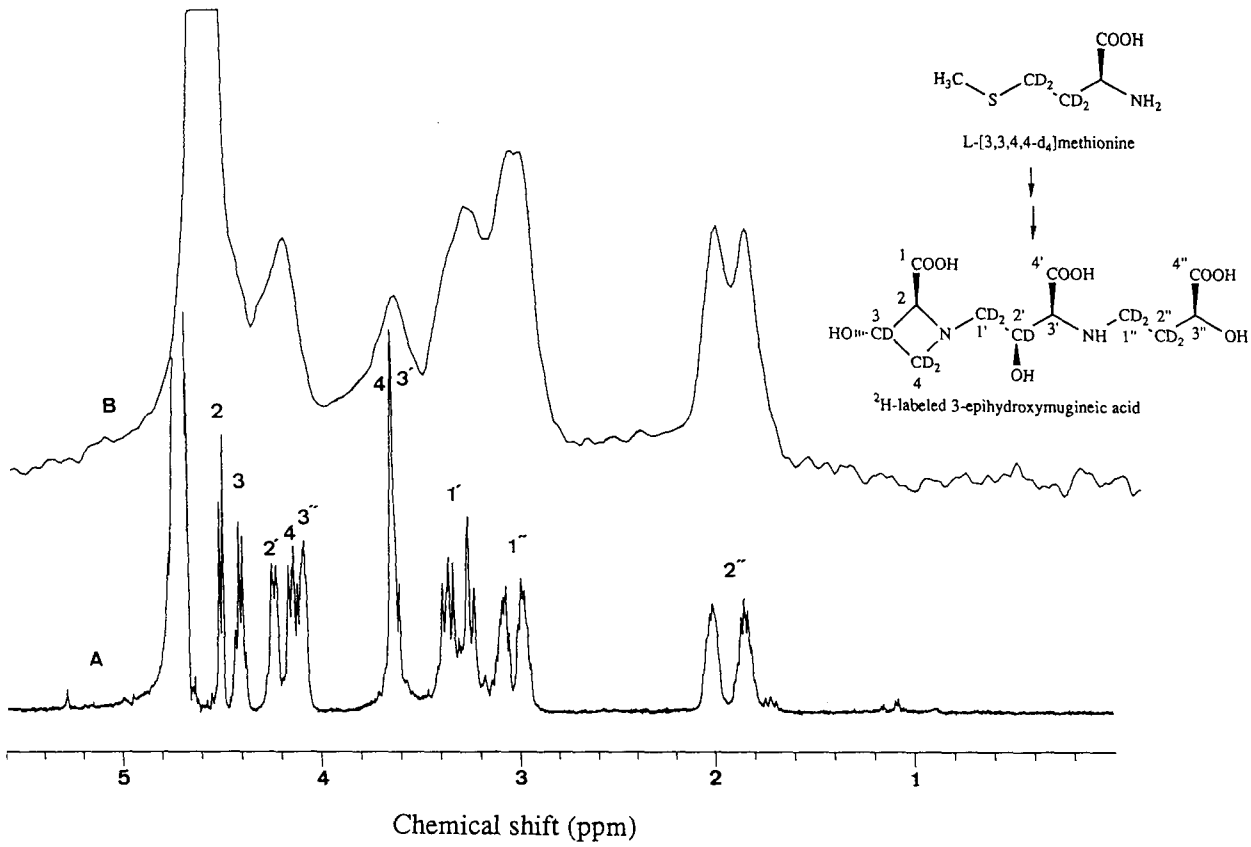

Figure 3. ^{1}H-NMR spectrum of unlabeled 3-epihydroxymugineic acid (A) and ^{2}H-NMR spectrum of 3-epihydroxymugineic acid biosynthesized from L-[3,3,4,4-d$_4$]Met (B) in beer barley. Refer to Figure 1 for spectral measurements. The peak at 4.70 ppm represents the signal of either H_2O or D_2O.

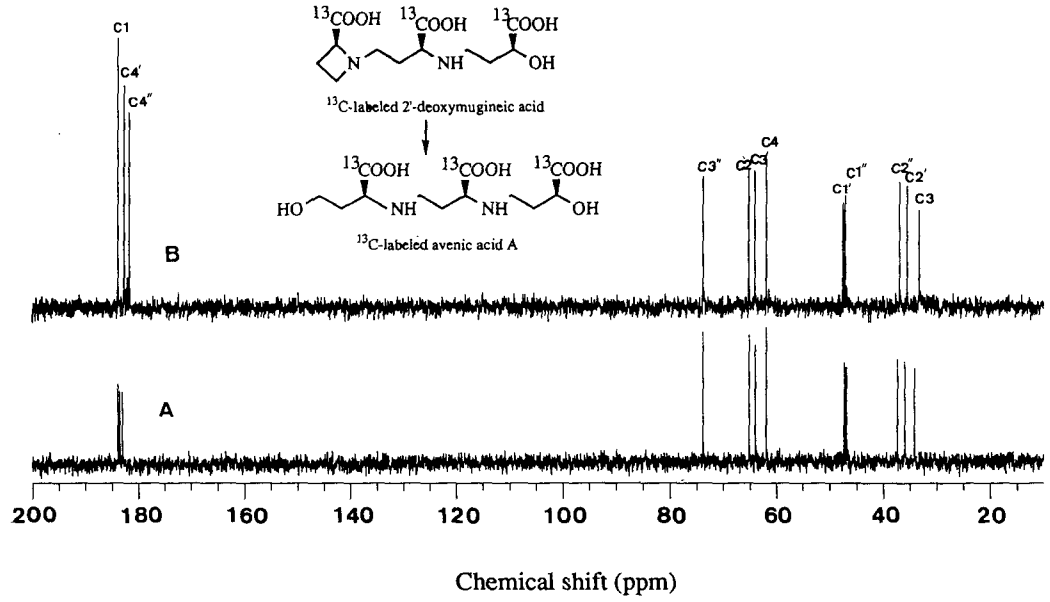

Figure 4. ^{13}C-NMR spectra of unlabeled avenic acid A (A) and avenic acid A biosynthesized from [1,4′,4″-$^{13}C_3$]2′-deoxymugineic acid (B) in oat. Spectra were measured with a 75.5-MHz spectrometer in 0.4 N ND_4OD.

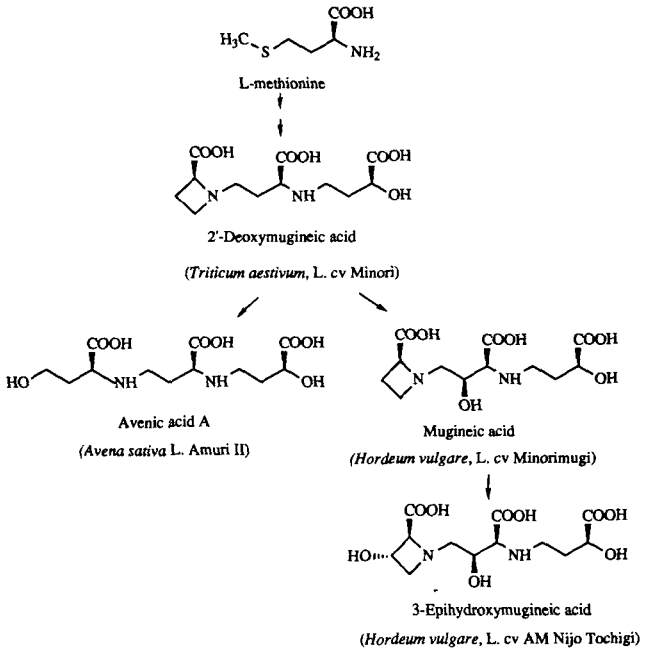

Figure 5. Two related biosynthetic pathways for different mugineic acids in gramineous plants.

during biosynthesis and relationships of different mugineic acids in their biosynthetic pathways would be clarified using this method.

When D,L-[3,3,4,4-d_4]Met was fed to gramineous plants, all isolated mugineic acids were ^{2}H-enriched by 10-fold. From root washings of wheat, 21.6 mg of labeled 2'-deoxymugineic acid was obtained. Relative peak intensities at 4.05, 3.93, 3.33, 3.15, 2.69, 2.50, and 2.12 ppm were approximated to 1:1:2:2:1:1:4 (Fig. 1B). According to ^{1}H-NMR assignment, these peaks corresponded to deuteriums at the C-4, C-1', C-1", C-3, C-2', and C-2" positions, respectively. Therefore, 12 deuteriums from 3 molecules of L-[3,3,4,4-d_4]Met were incorporated into the 2'-deoxymugineic acid.

The barley roots secreted 17.2 and 2.5 mg of labeled mugineic acid and 2'-deoxymugineic acid, respectively. The 2'-deoxymugineic acid showed a spectrum similar to that in Figure 1B. Eleven deuteriums were observed in the labeled mugineic acid whose relative peak intensities at 4.33, 3.94, 3.21, 3.13, 2.56, 2.46, 2.12, and 1.96 ppm were approximated to 1:2:2:2:1:1:1:1 (Fig. 2B). In contrast to the labeled 2'-deoxymugineic acid (Fig. 1B), one of the deuteriums at the C-2' position was lost in the labeled mugineic acid, suggesting that mugineic acid was derived from 2'-deoxymugineic acid by hydroxylation at the C-2' position.

Three labeled mugineic acids (2'-deoxymugineic acid, 1.9 mg; mugineic acid, 23.0 mg; 3-epihydroxymugineic acid, 1.6 mg) were isolated from the root washings of beer barley. The first two had spectra similar to those in Figures 1B and 2B, respectively. Relative peak intensities at 4.21, 3.65, 3.33, 3.09, 2.00, and 1.90 ppm were approximated to 2:1:2:2:1:1 in the labeled 3-epihydroxymugineic acid (Fig. 3B), corresponding thus to deuteriums at the C-2', C-4, C-1', C-1", and C-2" positions, respectively. One peak at 4.41 ppm was overlaid with an H_2O signal. The problem was alleviated by shifting the signal to a lower field by lowering the temperature to 10°C during NMR measurement (data not shown). This peak was assigned to the deuterium at the C-3 position. Unlike the labeled mugineic acid, one of the deuteriums at the C-3 position was lost in the 3-epihydroxymugineic acid.

Avenic acid A does not contain an azetidine ring in its structure. Mori and Nishizawa (1987) and Shojima et al. (1989) have previously speculated that 2'-deoxymugineic acid is biosynthesized from avenic acid A. However, the reverse is also possible; namely, avenic acid A may be produced from 2'-deoxymugineic acid by cleavage of the azetidine ring. In the present study, when D,L-[3,3,4,4-d_4]Met was fed to the iron-deficient oats, one of the deuteriums at the C-4 position was lost in the isolated avenic acid A (data not shown). However, 11 other deuteriums were incorporated in a manner resembling that of labeled 2'-deoxymugineic acid. Our data suggested that the precursor of avenic acid A was L-Met as 2'-deoxymugineic acid and that avenic acid A might be biosynthesized from 2'-deoxymugineic acid by cleavage of the azetidine ring. To confirm this, [1,4',4"-$^{13}C_3$]2'-deoxymugineic acid was fed to similar plants. The ^{13}C-NMR study revealed that the isolated avenic acid A (4.4 mg) was ^{13}C enriched by 2.6-, 2.3-, and 2.3-fold at the corresponding positions of C-1, C-4', and C-4", respectively (Fig. 4B). This, therefore, clarified that avenic acid A did not serve as a precursor for the 2'-deoxymugineic acid. Rather, the reverse was true; namely, avenic acid A was biosynthesized from 2'-deoxymugineic acid by opening the azetidine ring between the N and C-4 positions after hydroxylation at the C-4 position.

Results of the above ^{2}H- and ^{13}C-NMR studies reveal that L-Met serves as a precursor for all these mugineic acids. The present study provided the first direct evidence on relationships among the various different mugineic acids, especially the correlation between avenic acid A and 2'-deoxymugineic acid. Our findings suggested that there were at least two related biosynthetic pathways for mugineic acids (Fig. 5). Both these pathways shared the L-Met to 2'-deoxymugineic acid process. However, the subsequent steps differed and were dependent on the plant species. In oats, avenic acid A was biosynthesized from 2'-deoxymugineic acid by cleavage of the azetidine ring. In barley, hydroxylation at the C-2' position in 2'-deoxymugineic acid yielded mugineic acid. Further hydroxylation at the C-3 position in mugineic acid produced 3-epihydroxymugineic acid in beer barley.

So far, the intermediates between L-Met and 2'-deoxymugineic acid have not been isolated. Recently, nicotianamine has been reported as a possible previous precursor for 2'-deoxymugineic acid in the cell-free system (Shojima et al., 1990). Based on their structural features, nicotianamine might possibly be converted to 2'-deoxymugineic acid by deamination and subsequent reduction at the C-3" position, although direct verification is warranted. In the present study, when three molecules of L-[3,3,4,4-d_4]Met were incorporated into 2'-deoxymugineic acid, the number of deuteriums at the C-3 and C-4 positions in each molecule of Met did not change. This indicated that oxidation at the C-4 position in Met did not produce a viable intermediate. Isolations of

labeled intermediates between L-Met and 2′-deoxymugineic acid are now in progress in our laboratory.

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