Determination of Acetyl-CoA and Malonyl-CoA in Germinating Rice Seeds Using the LC-MS/MS Technique

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Received May 16, 2006; Accepted July 7, 2006; Online Publication, November 7, 2006
[doi:10.1271/bbb.60269]

A highly sensitive and selective analytical method was developed to determine levels of acetyl-CoA and malonyl-CoA in plant tissues. The analytical method includes a convenient extraction method for plant samples and a new LC-MS/MS technique utilizing an ion pair reagent. These acyl-CoAs, present in germinating rice seeds, were then determined by the method developed. It was found that the concentrations of both acetyl-CoA and malonyl-CoA increased with time during the germination of rice seeds and also increased at the elevated cultivation temperatures among tested. These results reflect the development of enzymes that produce these acyl-CoAs in germinating rice seeds.

Key words: acetyl-CoA; malonyl-CoA; LC-MS/MS; rice seed

Acetyl coenzyme A (acetyl-CoA) is an important biomolecule existing in many organelles and a precursor of fatty acid biosynthesis in organisms. Acetyl-CoA, used in fatty acid biosynthesis in plants, is mainly produced from pyruvic acid.1) Malonyl coenzyme A (malonyl-CoA) is a key compound in fatty acid biosynthesis and flavonoid biosynthesis. It is formed by carboxylation of acetyl-CoA, which is catalyzed by acetyl-CoA carboxylase (ACCase). Consequently, ACCase can be considered to be the starting enzyme of fatty acid biosynthesis, and it is the major site of the regulation of this anabolic pathway.2) Grass ACCase is the primary target site of the aryloxyphenoxypropionic acid herbicides and the cyclohexanedione herbicides.3–5) This enzyme is of two types in the cell, the prokaryotic type and the eukaryotic type. The above groups of herbicides, in vitro, inhibit ACCase of the eukaryotic type, but do not inhibit that of the prokaryotic type.6) In general, both types of enzymes exist in dicot plants. The grass family specifically does not have ACCase of the prokaryotic type, but has that of the eukaryotic type. This is the reason for herbicide selectivity between monocot and dicot plants.

Despite the importance of these metabolic processes and being the primary target of the grass herbicides, there is little knowledge of the levels of these acyl-CoA compounds in plant tissues due to lack of a suitable analytical method. Several reports have been published on measuring concentrations of acyl-CoA ester in rat tissues.7–9) Tumaney10) have reported concentrations of acetyl-CoA in several plant tissues. Post-Beittenmiller et al.11) have reported that malonyl-CoA was detected in chloroplasts incubated under light illumination, but they reported that it was difficult to measure accurately the concentration of malonyl-CoA because its level was extremely low in the chloroplasts.

High performance liquid chromatography (HPLC) is a conventional analytical method that has long been used as a main technique for determination of acyl-CoA esters.8,9,11) In these reports, however, short-chain CoAs in various tissues have been analyzed by different HPLC methods. These HPLC methods had longer run times and showed inadequate separation of some compounds, leading to misidentification and inaccurate quantification. Recently, HPCE (high performance capillary electrophoresis) was developed to deal with these difficulties.12) This method has a shorter analysis time and better separation than HPLC, but it requires specific analytical equipment that is not so general as HPLC. Additionally, HPLC and HPCE utilized a spectroscopic or fluorometric detector, which has low selectivity.

In this study, we established a new analytical method utilizing an LC-MS/MS technique that makes possible rapid and highly selective quantification of acetyl-CoA and malonyl-CoA in plant tissues containing many interfering materials. Using this method, we successfully monitored time course changes for concentrations of these compounds in rice seeds during germination at several cultivation temperatures.

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Abbreviations: ACCase, acetyl-CoA carboxylase; CoA, coenzyme A; DBAA, di-n-butylammonium acetate; HPCE, high performance capillary electrophoresis; HPLC, high performance liquid chromatography; MRM, multiple reaction monitoring; SPE, solid phase extraction; TFA, trifluoroacetic acid
Materials and Methods

Reagent. Acyl-CoA (acetyl-CoA, malonyl-CoA, and n-propionyl-CoA) standards were purchased from Sigma Chemical (St. Louis, MO) and di-n-butylammonium acetate (DBAA) and trifluoroacetic acid (TFA) were purchased from Tokyo Chemical Industry (Tokyo). All organic solvents used in this study were of HPLC or GC grade. The pure water used was prepared by a Milli-Q Gradient A10 system (Millipore, Bedford, MA).

Plant germination and sampling. Japonica rice seeds (Oryza sativa, Koshihikari) were supplied by the Japan Association for the Advancement of Phyto-Regulators. Cultivation was started by immersing sets of rice seeds in water in disposable plastic plates. One sample consisted of 10 seeds. The rice seeds were cultivated in an incubator (Tokyo Rikakikai, Tokyo) at three different temperatures (25, 30, and 35°C). Triplicate seed samples were harvested at six sampling times (0, 1, 5, 24, 48, and 72 h immersion) for subsequent analysis.

Determination of seed weight. The rice seeds were weighed immediately before cultivation and at each harvest. At each harvest, they were blotted dry with paper towels prior to extraction.

Sample extraction. The harvested rice seeds at each sampling point were immediately subjected to extraction (n = 3). The rice seed samples (0.2563–0.2890 g/10 seeds weight) were extracted for 1 min with an approximately 2.5 ml portion of 5% (v/v) TFA aqueous solution containing 50.8 pmol n-propionyl-CoA (an internal standard) using a Polytron homogenizer (Kinematica AG, Littau-Lucerne, Switzerland) under a chilled condition. The mixture was filtered through a filter paper. The filtrates were neutralized with a 25% aqueous ammonium solution. These solutions were rotary-evaporated to dryness. The residues were re-dissolved in 2 ml of 5 mmol DBAA and 5 mmol ammonium acetate buffer (pH 9.0) for the subsequent solid phase extraction. Other rice seeds were extracted with an approximately 2.5 ml portion of 5% TFA aqueous solution without n-propionyl-CoA to determine the background level of n-propionyl-CoA (n = 1) at all sampling points at the three different temperatures.

Solid phase extraction. Solid phase extraction (SPE) was carried out with a Sep-pak plus C18 (Waters, Milford, MA). The extracts were loaded on an SPE column and then eluted with a 6 ml portion of methanol. The eluate was rotary-evaporated at 40°C, and the residues were re-dissolved in a small amount of 0.1% acetic acid in water. The final solution was stored at 4°C until LC-MS/MS analysis.

LC-MS/MS method. LC-MS/MS was carried out using a LC-10ATvp HPLC system (Shimadzu, Kyoto) interfaced to a Q-Trap linear ion trap quadrupole LC-MS/MS mass spectrometer (Applied Biosystems, Foster City, CA). A Capcell Pak C18 MGII column (2.0 mm i.d. \times 150 mm, 5 μm), purchased from Shiseido (Tokyo), was used for HPLC separations. The following solvents were delivered at a flow-rate of 0.2 ml/min: (A) methanol, and (B) 5 mmol DBAA + 5 mmol ammonium acetate buffer, pH 9.0. The chromatograph was programmed to deliver the solvents as follows: initially 20% A, 80% B; a linear gradient to 15 min to 90% A, 10% B. The column oven temperature was 40°C. For quantification, the entire column effluent was introduced into the mass spectrometer. During LC-MS/MS analysis, the electrospray ion source of the mass spectrometer was operated at 550°C in negative ion mode to generate [M – H]− ions of the compounds. Following selection of the deprotonated precursor ions by the first quadrupole mass analyzer, collision-induced dissociation was carried out. Both the first and last quadrupole mass analyzers were operated at unit-mass resolution. During multiple reaction monitoring (MRM), the dwell time was set at 0.33 s for each of precursor/product ion pair. MRM-selected ions for acetyl-CoA, malonyl-CoA, and n-propionyl-CoA were 808.1/408.1, 852.1/408.1, and 822.1/408.1 respectively.

Quantification. n-Propionyl-CoA was used as an internal standard. A calibration curve was prepared daily by comparing the ratios of MRM peak areas of acetyl-CoA or malonyl-CoA versus those of n-propionyl-CoA. The measurement ranges of acetyl-CoA and malonyl-CoA were 0.094–46.974 pmol and 0.504–50.444 pmol respectively.

Results and Discussion

Development of analytical method

We developed the analytical method to determine the low concentrations of acetyl-CoA and malonyl-CoA present in the plant materials. The development of the analytical method included improvement of the extraction method and use of the MS/MS detector to detect the target compounds with high selectivity.

We selected a 5% TFA aqueous solution for the extraction solvent in this analytical method. Aqueous ammonia solution (25%) was used to neutralize the extracts. TFA is an acidic, volatile material and a suitable compound for LC-MS/MS analysis. Ammonia is also a volatile material. It is known that the analytes, acyl-CoAs, are comparatively stable in acidic solvent.7) Conventionally, the extraction of these acyl-CoAs from the various materials has used trichloroacetic acid or perchloric acid aqueous solution.8,9) These extraction solvents generate a large amount of nonvolatile salts, which are not appropriate to LC-MS/MS analysis, during their neutralization procedures. In our experiment, the extracts after neutralization were loaded on the
solid phase extraction to remove those proteins that can interfere with LC-MS/MS analysis.

Figure 1 shows MS/MS spectra and MRM chromatogram of three standard solutions of acyl-CoAs. We prepared an HPLC solvent that contained ammonium acetate and DBAA. DBAA is an ion pair reagent available for the LC-MS detector and is suitable to form ion pairs with acyl-CoAs. The HPLC method used in this experiment improved the retention of the HPLC column for acyl-CoAs compounds. Since the HPLC methods that have been reported used HPLC solvent containing acid or buffer solutions, the retention of the HPLC column for acyl-CoAs was insufficient.7–9,11) We selected the MS/MS detector. The extracts in our experiment included many interfering materials due to the simple extraction procedure. The MS/MS detector accurately determined the acyl-CoAs in these extracts at lower concentrations. The analytical method in the previous reports used a UV detector, which had low selectivity.7–9,11) The detection limits of acetyl-CoA and malonyl-CoA reported were 50 pmol and 80 pmol respectively.12) Recently, an LC-MS analytical method that uses a stable isotope of malonyl-CoA was developed.13) The detection limit of malonyl-CoA by this method was 50 pmol. The detection limits of our method are 0.1 pmol (acetyl-CoA) and 0.5 pmol (malonyl-CoA). Hence, our method can determine these acyl-CoAs with one hundred times higher sensitivity than the previous method.

We selected n-propionyl-CoA as an internal standard.

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Fig. 1. MS/MS Spectra and MRM Chromatogram of Acyl-CoAs.
A, MS/MS spectrum (precursor ion scan of m/z 808) and chemical structure of acetyl-CoA. B, MS/MS spectrum (precursor ion scan of m/z 852) and chemical structure of malonyl-CoA. C, Typical MRM chromatogram of acetyl-CoA, malonyl-CoA, and n-propionyl-CoA in calibration solution. Injection amounts of acetyl-CoA, malonyl-CoA, and n-propionyl-CoA were 47.0 pmol, 50.4 pmol, and 50.8 pmol respectively.
In the first experiment, \( n \)-propionyl-CoA in the rice seeds were determined at all sampling points at the three different temperatures without the addition of the internal standard \((n = 1)\). This compound was not detected in the extracts, except for rice seeds of 96 h cultivation at 35°C. The peak level was about nine-fold higher than the background level (Fig. 2A). The detected level was about 1/50 lower than the peak level in the extracts in which the internal standard was added (Fig. 2B). These results indicate that \( n \)-propionyl-CoA was suitable as the internal standard.

The recovery experiment for our method was done by spiking a known amount of acetyl-CoA and malonyl-CoA using intact rice seeds. These recoveries were 102.8% for acetyl-CoA and 89.6% for malonyl-CoA. In conclusion, it became possible to determine lower concentrations of acetyl-CoA and malonyl-CoA produced in the rice seeds under germination. As a next step, we applied the new method in the next experiment.

**Determination of acetyl-CoA and malonyl-CoA in germinating rice seeds**

Figure 3 shows the time-course increases of germinating seed weights as a function of temperature. Seed weights were measured at six sampling points at the three temperatures. It was observed that seed weights increased in the first 1 h at the three different temperatures. The highest increase was found at 35°C.

Figure 4 shows the time-course increases of the acetyl-CoA concentration in germinating rice seeds as a function of temperature. The acetyl-CoA concentration in the rice seeds before cultivation was 6.9 pmol/g (sample weight). The concentration of this compound in the rice seeds was increased by longer cultivation periods and higher cultivation temperatures. The concentration of acetyl-CoA in the rice seeds at 96 h cultivation was 700 pmol/g at 25°C, 1,027 pmol/g at 30°C, and 1,135 pmol/g at 35°C. Acetyl-CoA is a central metabolite in a variety of important physiological processes with links to anabolism and catabolism, including fatty acid biosynthesis. It has been reported that pyruvate dehydrogenase in the generation route
from pyruvic acid to acetyl-CoA plays a predominant role in Arabidopsis seeds. The increment of acetyl-CoA concentration in the rice seeds in the first 1 h of cultivation suggested that the biological reaction of pyruvate dehydrogenase occurred immediately. We observed the highest increase of acetyl-CoA in rice seeds at the highest temperature.

Figure 5 shows the time-course increases in malonyl-CoA concentration in germinating seeds as a function of temperature. This compound was not detected before cultivation or after 1 h of cultivation at 25°C. The concentrations of malonyl-CoA in the rice seeds ranged from 9.5 pmol/g at 1 h (30°C) to 322 pmol/g at 96 h (35°C). The increment pattern of malonyl-CoA concentration in the rice seeds were the same as that of acetyl-CoA concentration. Malonyl-CoA is a key compound in fatty acid biosynthesis. It is formed by carboxylation of acetyl-CoA, which is catalyzed by acetyl-CoA carboxylase. We observed the highest increase in malonyl-CoA in the rice seeds at the highest temperature.

Tumaney et al. reported that the concentration of acetyl-CoA in several plants ranged from 5 to 25 nmol/g (fresh weight). Post-Beittenmiller et al. reported that it was difficult to measure accurately the concentration of malonyl-CoA in chloroplasts. This is the only report describing the concentration of malonyl-CoA in plant tissues. Our method made it possible to determine lower concentrations of acetyl-CoA and malonyl-CoA in plant tissues. As Figs. 3, 4, and 5 indicate, the increment of seed weight correlated closely with that of the concentration of acetyl-CoA and malonyl-CoA in it. These acyl-CoA compounds are the starting biomolecules in plant fatty acid biosynthesis. Fatty acids are an essential constituent of all plant cells. Our results indicate that the concentration of these acyl-CoAs compounds increased in the plant tissues because plant cell growth requires fatty acids. It will be necessary to determine the concentrations of acyl-CoAs compounds in plants under various cultivation conditions to understand these compounds in relation to the cultivation environment.

Acknowledgments

The authors thank Ms. Yukiko Nagao for technical assistance with plant cultivation and extraction.

References

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