J. Appl. Biol. Chem. **50**(4), 211-214 (2007) **Article**

Quantitation of Formate in Plants and Its Enhancement in Response to Environmental Stresses

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Received April 4, 2007; Accepted October 30, 2007

A solid-phase microextraction and gas chromatography-mass spectrometry utilizing ^{13}C-formate as an internal standard for the determination of formate was proved to be applicable as a reliable quantitative method in several plants. We were the first to discover that trees contain larger pool sizes of formate than herbs. Our data also showed that the formate level of the leaves increased after the methanol-spraying, suggesting that methanol oxidation could convert formaldehyde into formate. In addition, drought and chilling led to the increase of endogenous formate in *Arabidopsis thaliana*. These results confirmed that formate is a universal stress signal in plants.

Key words: *formate, formate dehydrogenase, environmental stress, methanol*

Plants contain small, metabolically active pools of formate and various pathways to produce formate have been proposed [Igamberdiev *et al.*, 1999]. In the green tissues of C_3 plants in the light, formate is generally considered to be produced from the photorespiratory pathway through a nonenzymatic, H_2O_2-dependent decarboxylation of glyoxylate. Other possible sources of formate synthesis include oxidation of formaldehyde and cleavage of 10-formyl-tetrahydrofolate. The formate can be an important source of C_1 units. Radioisotopic studies have shown the ready incorporation of ^{14}C-labeled formate into serine, methyl groups, and other products of the folate metabolism [Wingler *et al.*, 1999]. ^{13}C NMR measurements demonstrated a substantial flux from the supplied formate to serine in *Arabidopsis* [Hanson and Roje, 2001]. The importance of these pathways is not entirely clear, but one of the possibilities is the homeostatic regulation of the formate level. Formate may be an important substrate for the oxidation in mitochondria. A switch to the oxidation of formate is observed especially under stress conditions such as darkness, hypoxia, wounding, cold, and drought [Hourton-Cabassa *et al.*, 1998]. Shiraishi *et al.* (2000b) suggested that formate is involved in the endogenous radical-scavenging and/or in the supply of CO_2 derived from the formate, thereby reducing the oxidative damages to the photosystems under the photoinhibitory conditions. In addition, Shiraishi *et al.* (2000a) showed that the application of formate (1.8 mM) to the roots of rice seedlings leads to the growth increment both in terms of the length of the aerial part and the fresh weight. However, Li *et al.* (2002) suggested that exposure of 8 mM formate to *A. thaliana* turns the plant yellow and small, and markedly inhibited the primary root elongation. What then is the role of formate metabolism in plants? Up to date, very little is known about the origin, contributions and roles of formate in the plant budget. In particular, the physiological significance of the formate endogenous variation in plants under various environmental alterations is still unclear. Therefore, to investigate the effects of environmental stress on the formate content of the plant and thus infer the physiological role of formate in the plant metabolism, the changes in the formate endogenous level of *A. thaliana* under various stress conditions were investigated in this study.

For quantification, precise analysis of the metabolite is the most important factor. In our previous study, we already established a feasible method for the analysis of formate by solid phase microextraction (SPME) and GC-MS using ^{13}C-formate as an internal standard (I.S) [Kim

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et al., 2003]. Bearing in mind that an internal standard should match the chemical and physical properties of the compound of interest as closely as possible, a stable isotope-labeled form of the analyte to be measured is perfectly suitable as an I.S [Kim *et al.*, 2005]. However, mass spectrometry is not without problems related to the interference, especially through the matrix effects. Thus, whenever mass spectrometric assays are developed, matrix effects studies should also be performed using the sample extracts [Annesley, 2003].

To experimentally verify the quantification of formate contents of the plants utilizing SPME-GC-MS analysis, we analyzed the pool sizes of the formate in various plants and changes in the formate contents of the leaves of *A. thaliana* under various stress conditions.

Materials and Methods

Plant material. *A. thaliana*, *Oryza sativa*, and *Phaseolus vulgaris* were grown in a growth cabinet with light/dark period of 16/8 h at a photon flux density (PFD) of 100 μmol · m^{-2} · s^{-1} at 23°C for 25 d. The other plant species used for this study were sampled in Osaka University, Suita City, Osaka, Japan. The plants investigated were: *Solidago altissima, Equisetum arrense, Setaria viridis, Phaseolus yedoensis, Ginkgo biloba, Acer palmatum, Abelia grandiflora,* and *Hedera helix*. Leaves of the plants were harvested at midday by plunging them into the liquid nitrogen and were stored at −80°C until assayed.

Quantification of formate in plant. The young leaves of 25-d-old seedling were disrupted in liquid nitrogen, and the aliquot (40 mg) was overlaid with 1 mL of 200 mM KOH solution containing 50 μM ^{13}C-formate. The sample was lysed by vortexing for 30 s and centrifuged at 15,000 × g for 10 min at 4°C, and the supernatant was then used for the analysis of the formate. After derivatization with methyl formate under the acidic conditions, SPME-GC-MS procedures were performed according the method of Kim *et al.* (2003).

Calibration curve and matrix effects. The formate calibration standard consisted of 5-200 μM of non-labeled formtae and 50 μM of ^{13}C-sodium formate as I.S. These solutions were stored at −20°C.

Moreover, to assess the ^{13}C enrichment in the non-labeled formic acid, the ^{12}C-formate and ^{13}C-formate concentrations in 0.5 mL of distilled water were varied from 0 to 100 μM at eleven different concentrations. The mole-percentage of ^{13}C-enrichment (MPE) was calculated according to the equation (C_{tot}: sum of ^{12}C- and ^{13}C-formate concentration, C_{13C}: ^{13}C-formate concentration):

$$MPE\ (\%) = C_{13C}/C_{tot} \times 100$$

To evaluate the matrix effects on the signal intensity, the extracts of *A. thaliana* were used. After various concentrations of ^{12}C-formate were added (25, 55, 100, 150, and 185 μM) into the *A. thaliana* extracts, the extracts were centrifuged at 15,000 × g for 10 min. The formate contents of the supernatants were measured by the SPME-GC-MS procedure. The detected ^{12}C-formate values in the plant extracts that had been spiked with various concentrations of ^{12}C-formate were compared with those of ^{12}C-formate standard solutions.

Environmental stress treatments. Changes in the formate contents in the leaves of *A. thaliana* were analyzed under various stress conditions. To induce drought stress, 25-d-old plants were dug up carefully and placed for 12 to 24 h on a sheet of Parafilm. Cold stress was induced at 4°C for 12 to 24 h. Chemical treatments were performed by spraying the plants with 20% MeOH or 100 μM methyl viologen for 12 h. These stresses were performed under the same photoperiod as the control plants. The *A. thaliana* leaves were harvested and immediately frozen in liquid nitrogen for the formate assay. Formate contents were analyzed following the previous formate assay procedure (SPME-GC-MS analysis).

Results and discussion

Method validation. In our previous studies, the headspace SPME-GC-EI-MS-SIM with ^{13}C-formate as I.S. was more accurate and sensitive than the previously reported methods. Figure 1 shows a linear standard curve of ^{13}C-

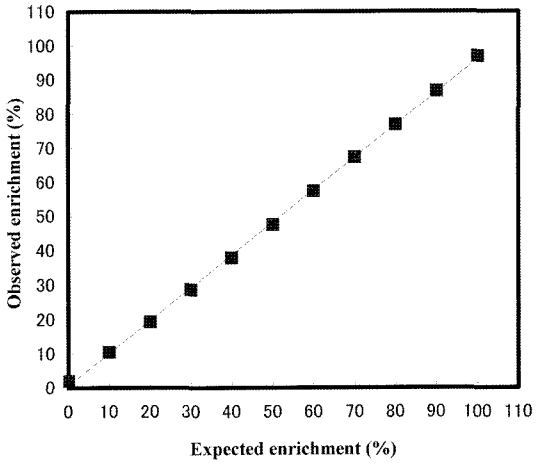

Fig. 1. Standard curve of ^{13}C-formate enrichment. ^{12}C- and ^{13}C-formates were converted into ^{12}C- and ^{13}C-methyl formates, respectively, which were assayed using the SPME-GC-MS method through the electron impact mass spectrometry. The proportions of ^{13}C- and ^{12}C-formates at 100 μM varied. Enrichment (%) represents the percentage of ^{13}C-formate in the total (^{12}C- and ^{13}C-) formates of 100 μM.

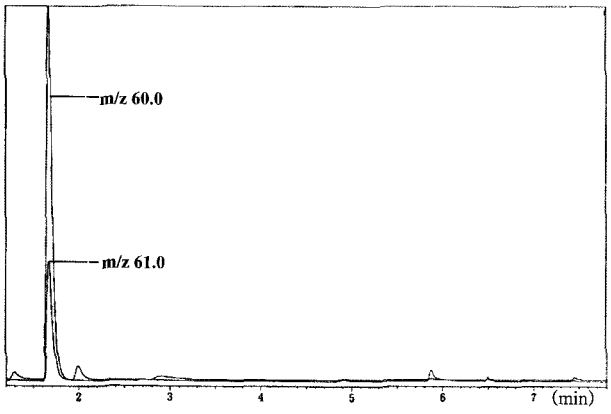

Fig. 2. Selective ion chromatogram of formate extracted from *Arabidopsis thaliana* leaves.

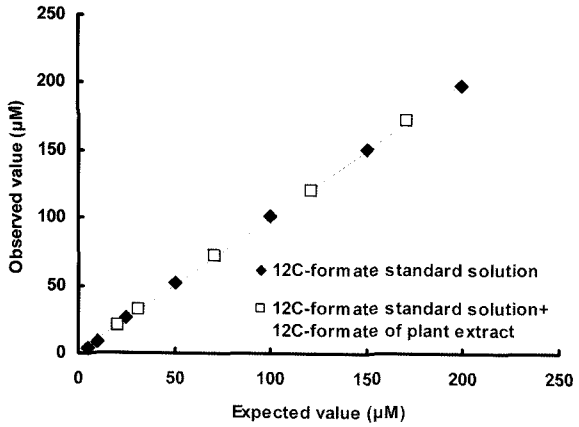

Fig. 3. Observed concentrations of ^{12}C-formate standard and ^{12}C-formate added to the plant extract. The calibration curve of the standard solution overlapped with that of the formate added to the plant extract.

formate enrichment. We obtained an excellent linear correlation between the expected enrichment and the detected enrichment, which was calculated from *m/z* 61 and 60 values. The slope, *r* value, and intercept were 0.953, 0.9995, and 0.6099, respectively. To test the applicability of the proposed method to the real samples, the content of formate in *A. thaliana* was determined. Selective ion chromatogram (*m/z* 60 and 61) of the formate extracted from *A. thaliana* leaves and ^{13}C-formate are shown in Fig. 2. The content of formate in the leaves was 1.10 ± 0.02 μmol/g (fresh weight).

Next, the *A. thaliana* extracts were spiked with the formate to examine the matrix effects of the established SPME-GC-EI-MS-SIM method. As expected, the observed value of the plant extract, which had been spiked with various concentrations of ^{12}C-formate, showed the highly consensus expected value. The standard curve of ^{12}C-formate value of the *Arabidopsis* extracts overlapped with that of the ^{12}C-formate standard solutions. The ratio

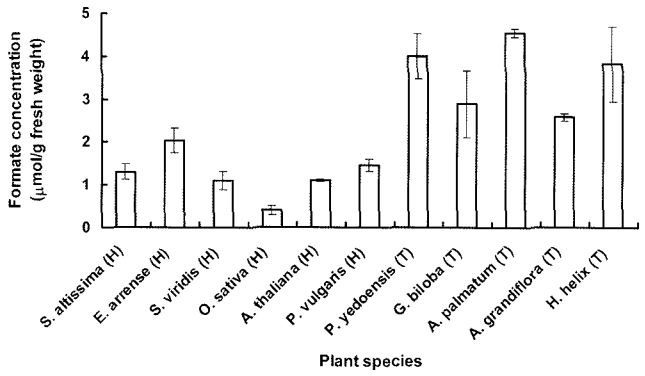

Fig. 4. Formate contents of various plant leaves. Data are means of three plants ± SE. *S. altissima, Solidago altissima; E. arrense, Equisetum arrense; S. viridis Setaria viridis; O. sativa, Oryza sativa; A. thaliana, Arabidopsis thaliana; P. vulgaris, Phaseolus vulgaris; P. yedoensis, Phaseolus yedoensis; G. biloba, Ginkgo biloba; A. palmatum, Acer palmatum; A. grandiflora, Abelia grandiflora; H. helix, Hedera helix*. Herb and tree are designated as H and T, respectively.

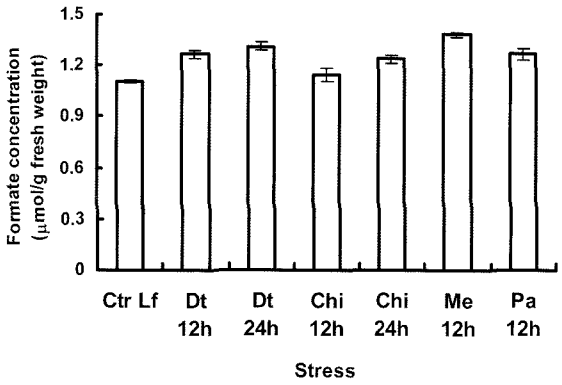

Fig. 5. Formate contents of *Arabidopsis* leaves after various stress treatments. Data are means of three plants ± SE. Ctr Lf, Control leaves; Dt 12 h and Dt 24 h, 12 and 24 h of drought treatments, respectively; Chi 12 h and Chi 24 h, 12 and 24 h of chilling, respectively; Me 12 h, 12 h after spraying 20% methanol; Pa 12 h, 12 h after spraying paraquat.

of the measured formate to the increasing amount of the formate was linear with an *r* value of 0.9995. The within-run assay coefficients of the variation (n = 3) for the formate levels were all less than 1.5%. The equations for the curve were: $y = 0.0202\chi + 0.3562$ for plant extracts, and $y = 0.0201\chi + 0.0854$ for the ^{12}C-formate standard solution (Fig. 3).

The pool sizes of the formate in various plants were also identified; all plants contained small pool of the formate, and the formate contents differed among plants (0.5 to 4.0 μmol/g fresh weight) (Fig. 4). In addition, the formate contents in the leaves of the trees were higher than those of the herbal plants.

Changes in the endogenous level of formate following the imposition of stresses. We found for the first time that the drought, chilling, and chemical stress increased the formate content (Fig. 5). How is formate produced in the plants? In the *Arabidopsis* cell culture after the salt stress treatment, we previously suggested that the formate accumulation may be due to the glyoxylate decarboxylation caused by the deficiency of the amino donors required for the transamination of glyoxylate during photorespiration [Kim *et al.*, 2007]. Also, Formate oxidation has been noted in the anaerobiosis and the suppression of heme synthesis caused by iron-deficiency [Suzuki *et al.*, 1998]. Under various stress conditions, the transcript level of FDH increased, whereas that encoding serine hydroxymethyltransferase (SHMT) decreased [Hourton-Cabassa *et al.*, 1998]. Therefore, it could be hypothesized that various stresses lead to the production of formate, which is oxidized into CO_2 by formate dehydrogenase (FDH), but not utilized in the SHMT reaction. Under stress, the formate respiration may complement the classic respiration, especially when the Krebs cycle is not fully operative.

Of particular interest is the accumulation of formate after spraying methanol on the leaves. Both the cell wall growth and degradation lead to the methanol production in plants [Fall and Benson, 1996]. The methanol-treated plants exhibited very high levels of FDH mRNA [Hourton-Cabassa *et al.*, 1998]. Shiraishi *et al.* (2000a) previously suggested that the exogenous potassium formate is quickly taken up by rice and converted into CO_2 by FDH. Formate is a good candidate for the final signal in the transduction pathways leading to the FDH response, and the abundance of the enzyme transcripts is also strongly increased under various stress conditions such as drought, hypoxia, chilling, dark, and wounding [Hourton-Cabassa *et al.*, 1998]. Thus, our results postulate that formate can be a universal stress signal in plants, and the excess formate produced during the environmental alterations may be oxidized by FDH.

In conclusion, the SPME-GC-EI-MS-SIM method was proved to be applicable for the evaluation of the formate concentration in the plant tissues. The formate level was enhanced after various stress treatments, suggesting that the formate may be a universal stress signal in plants.

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