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Folate Levels and Polyglutamylation Profiles of Papaya (*Carica papaya* cv. Maradol) during Fruit Development and Ripening

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Supporting Information

ABSTRACT: Folates are essential micronutrients for humans, and their deficiency causes several detrimental effects on human health. Papaya fruit is an important natural source of some micronutrients. This paper presents a first complete characterization of folate derivatives accumulated in cv. Maradol papaya during fruit development and ripening processes. During postharvest ripening, the fruit accumulated up to 24.5% of the daily folate recommended dietary allowance (RDA) for an adult in a 1 cup (145 g) portion. Tetrahydrofolate (THF) and 5-methyl-THF were the predominant folate classes observed. Surprisingly, an unusually long polyglutamylation profile of tentatively up to 17 glutamates linked to 5-methyl-THF was detected; to the authors' knowledge, this very long polyglutamyl tail has not been reported for any organism, and it is probably characteristic of this plant species. This polyglutamylation degree changed throughout fruit development and ripening, showing the largest differences at the onset of ripening. This work raises questions about the functional role of folate derivatives in fruit development.

KEYWORDS: *folates, polyglutamyl tail, papaya, fruit ripening, vitamin, postharvest quality*

INTRODUCTION

Papaya (*Carica papaya* L.) is a tropical plant that belongs to the Caricaceae family, and it is an important fruit crop produced mainly in Latin America, Asia, and Africa. Its fruit is consumed worldwide fresh and processed into juices and jams. Papaya fruit is recognized to have high nutritional value; it is an excellent source of provitamin A, carotenoids, and ascorbate (vitamin C) and is also considered a good source of α-tocopherol and folates (vitamin B9). Recent works have characterized carotenoid, ascorbate, and phenolic profiles in the fruit mesocarp to obtain a better knowledge of its nutritional composition.[1−3] However, individual folate profiles have yet to be characterized, because only total folate contents have been reported in nutritional tables for the fruit at mature stage.[4,5]

Folate is the collective term that groups tetrahydrofolate (THF) and its derivatives. Folates are needed as cofactors for the enzymatic one-carbon transfer reactions in organisms, generally known as 1C metabolism; these reactions are involved in the biosynthesis of methionine and nucleic acids and the interconversion of glycine and serine.[6] Folate consumption is essential for human health; humans are not able to produce folates and have to obtain them from the diet. Folate deficiency elevates the risk of births with neural tube defects and has been linked with other pathologies that include cancer and cardiovascular disease.[7] Chemically, the THF structure is composed of a pteridine ring attached to a *p*-aminobenzoate (PABA) that is also linked to a glutamyl tail (Figure 1A). Nitrogens 5 and 10 in the pteridine ring can contain a transferable carbon group at different oxidation states. Folate derivatives are classified depending on this 1C substitution and on the number of glutamates within the polyglutamyl tail. The oxidation state of the carbon, in addition, influences the stability of the molecule (THF is the most labile class and 5-formyl-THF the most stable natural folate).[8] Additionally, the polyglutamylation extent of the molecule can be an important factor for both stability and bioavailability of the vitamin. Folates attached to proteins are more stable, and some folate-utilizing enzymes have more affinity for polyglutamylated folates.[6,9] On the other hand, after consumption, polyglutamylated folates need to be enzymatically deglutamylated to their monoglutamyl form to be absorbed; if this step is not completed, their bioavailability is affected.[10] Because papaya fruit is considered a good folate source on the basis of its total folate levels (54 μg per portion[4]), we considered it important to study its folate profiles and how they change during postharvest fruit maturation.

Mexico is one of the main worldwide papaya producers, and the red-fleshed cultivar Maradol represents >90% of the planted papaya in the country.[11,12] We chose then to analyze Maradol fruit, which has been already studied for its high β-carotene and lycopene accumulation and is widely consumed.[1] The present work describes the characterization of folate classes in papaya mesocarp during fruit development and postharvest ripening.

MATERIALS AND METHODS

Plant Material. Three developmental and five fully developed ripening stages distinguished by their skin and flesh colors were considered in the present work[13] (Figure 1B). Fruit and leaves were obtained during April 2010 from *C. papaya* cv. Maradol young plants (11-month-old, second harvest) from a commercial plantation localated in Cohetzala, Puebla, Mexico. Three different developmental stages (0.3, 0.5, and 0.8 of the full fruit size) and fully developed ripening stages 1 and 2 (Figure 1B) were harvested directly from the plants. Fruit and leaves were flash-frozen with liquid N_2 and kept at

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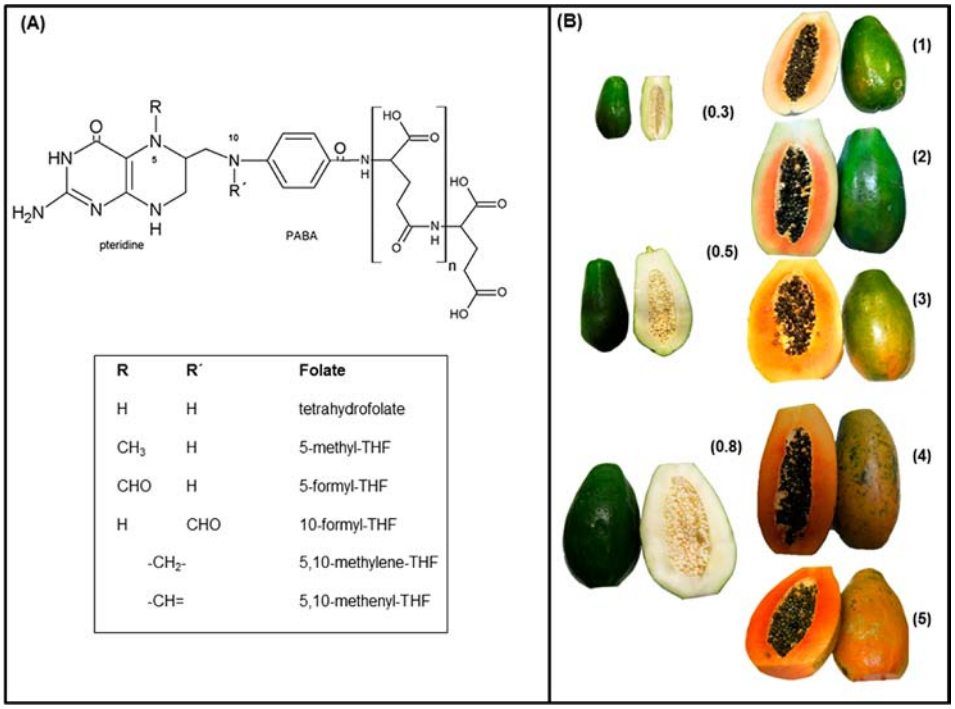

Figure 1. Chemical structure of polyglutamylated folates (A) and papaya fruit developmental and ripening stages analyzed in this work (defined according to ref 13) (B). Developmental stages are defined by the fraction size of the fully developed fruit; ripening stages are defined as the number of color stripes on fruit skin and the depth of flesh red color. PABA, *p*-aminobenzoate.

−80 °C, and some fruit at the 1 and 2 ripening stages were used for a controlled postharvest ripening study. Harvested fruit was treated with 1 g/L of fungicide Promyl (Promotora Técnica Industrial, Morelos, Mexico) following the instructions of the manufacturer. Postharvest ripening was achieved by storing stage 2 fruit at 21 °C for a maximum of 9 days, sampling at different times when fruit reached each ripening stage. One sample for physicochemical and folate analyses consisted of a pool of longitudinal slices from five papayas that were homogenized using a Waring 2.5 qt food processor (Waring, New Hartford, CT, USA) after they were frozen with liquid N_2; the homogenized samples were kept at −80 °C until analysis. Yellow-fleshed papaya used for comparison purposes was obtained at the market at ripening stage 3, sliced, flash-frozen, and kept at −80 °C.

Physicochemical Characterization. Total solid contents of the fruit were measured following AOAC method 920.151.[14] The pH of the macerated flesh was determined with a properly calibrated Orion 3Star pH benchtop potentiometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Total soluble solids were determined using an Atago HSR-500 refractometer (ATAGO Co., Tokyo, Japan), and results were expressed in °Brix according to the AOAC 932.12 method.[14]

Folate Analysis with Electrochemical Detection. Folates were extracted and purified as described previously.[15] Briefly, the homogenized tissue (1 g) was dissolved in extraction buffer (50 mM HEPES, 50 mM CHES, 1% ascorbate, and 10 mM *β*-mercaptoethanol) with $CaCl_2$ (4 mM) added for pectin precipitation and boiled for 10 min for folate release by protein denaturation. Half of the extract was treated with a recombinant conjugase from *Arabidopsis* for folate deglutamylation[16] for 1 h at 37 °C. Folates were purified by affinity chromatography using folate-binding columns made of agarose (Affigel, Bio-Rad, Hercules, CA, USA) linked to a folate binding protein obtained from milk whey. Purified folates were separated by HPLC (Agilent Technologies, Santa Clara, CA, USA) using an Atlantis dC18 column (150 mm × 4.6 mm; 5 *μ*m particle size) (Waters, Milford, MA, USA) for maturation analysis and a Prodigy ODS(2) column (150 mm × 3.2 mm; 5 *μ*m particle size) (Phenomenex, Torrance, CA, USA) for leaves and yellow-fleshed papaya. Separation was achieved with a 55 min nonlinear gradient of phase A (28 mM K_2HPO_4, 59 mM H_3PO_4) and phase B (75% phase A, 25% acetonitrile): 10% B (0−10 min), 10−30% B (10−20 min), 30−80% B (20−50 min), 100% B (50−55 min) at 1 mL/min. Folate classes were detected with a four-channel electrochemical detector (CoulArray, ESA, Chelmsford, MA, USA) with potentials set at 100, 200, 300, and 400 mV in each channel. Calibration curves were made using THF, 5-methyl-THF (5-CH_3-THF), 5-formyl-THF (5-CHO-THF), and 5,10-methenyl-THF (5,10-CH=THF) standards obtained from Schircks (Shircks Laboratories, Buechstrasse, Jona, Switzerland). 5-CH_3−THF tetraglutamylated and folic acid heptaglutamylated were obtained also from Schircks Laboratories. 5-CH_3-THF heptaglutamic acid (Glu7) was synthetized from pteroylhepta-*γ*-L-glutamic acid ammonium salt following the procedure described by Selhub.[17] Both polyglutamylated forms of 5-CH_3-THF were used to confirm polyglutamylated peaks found in papaya.

Folate Analysis with Fluorescence Detection. To confirm to which folate form the polyglutamylated chains were linked, non-deglutamylated folate extracts from papaya fruit (6 g, Maradol and yellow-fleshed) were analyzed with an HPLC with a fluorescence detector (Waters) using a Prodigy ODS(2) column (see above). Folate extraction, purification, mobile phases, and gradient were conducted as explained previously. Folate detection was made at 295 nm excitation and 358 nm emission wavelengths. Under these conditions, 5-CH_3-THF has 100% of relative fluorescence response, whereas the other folate classes have <20% fluorescence response relative to 5-CH_3-THF.[18]

RESULTS AND DISCUSSION

Physicochemical Properties of Papaya Fruit during Development and Ripening. The moisture contents of the fruit ranged from 87.9 to 92.4% during all developmental and ripening stages (Table S1, Supporting Information). This small range of variation did not represent a significant impact for the assessment of folate levels; therefore, we present all measurements in fresh weight. Total soluble solids increased as fruit ripened, and pH values measured in mesocarp ranged from 5.1 to 5.6, as reported by others.[13] These measurements indicate

Table 1. Folate Contents during Fruit Development and Ripening[a]

developmental and ripening stage	folate content (pmol g^{-1} fresh weight)				
	THF + 5,10-CH_2-THF	5-CH_3-THF	5,10-CH=THF + 10-CHO-THF	5-CHO-THF	total folates
Fruit Development					
0.3	493 ± 42 bcd	305 ± 17 d	72 ± 27 bc	41 ± 16 a	911 ± 82 bc
0.5	461 ± 113 cd	317 ± 68 d	65 ± 12 bc	5 ± 1 b	848 ± 171 c
0.8	635 ± 43 b	349 ± 8 cd	91 ± 34 ab	7 ± 1 b	1082 ± 38 b
Ripening Stages					
1	434 ± 37 cd	441 ± 44 b	55 ± 20 cd	36 ± 13 a	966 ± 73 bc
2	406 ± 28 d	415 ± 18 bc	59 ± 12 bc	40 ± 13 a	919 ± 69 bc
3	905 ± 283 a	680 ± 59 a	22 ± 6 d	44 ± 9 a	1651 ± 210 a
4	573 ± 85 bc	449 ± 52 b	24 ± 7 d	26 ± 3 a	1072 ± 114 b
5	965 ± 50 a	361 ± 21 cd	106 ± 10 a	29 ± 7 a	1461 ± 76 a

[a]Different letters in the same column are significantly different (LSD test, $p < 0.05$). All values are the mean ± SD of three independent determinations.

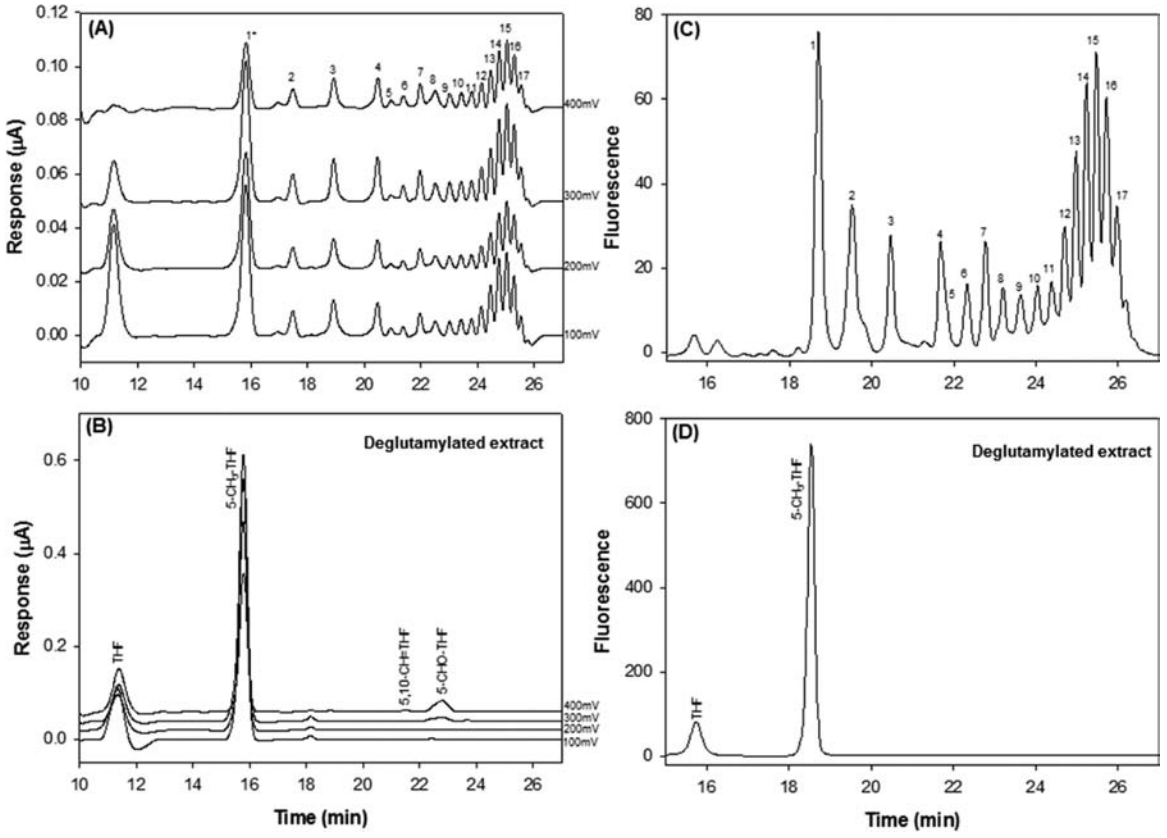

Figure 2. Chromatographic profiles of poly- and monoglutamylated folates in papaya fruit in two detection systems. Chromatograms of polyglutamylated folate extract (A) and conjugase-treated extract showing hydrolyzed folates to their monoglutamyl form (B) using the electrochemical detector. Each folate class has a specific response in each channel set at different potential; examination of this response plus the disappearance of the peak in the deglutamylated profile helps to discriminate signals as polyglutamylated folates. Chromatograms of poly- (C) and monoglutamylated (D) folate extract were detected by a fluorescence detector. Note the change in the scale of the *y* axis for poly- and monoglutamylated profiles.

that the ripening process occurred normally during the postharvest storage. Fruit usually is commercially acceptable for consumption at ripening stages 4 and 5.

Folate Contents in Papaya Fruit during Development and Ripening. Total folate accumulation during development and the first two ripening stages showed no significant changes in papaya fruit (Table 1). On the other hand, we found a slight, significant increase in folate accumulation at late ripening (stages 3 and 5). The total amount of folates measured in this study were higher than those reported in nutritional tables (176%).[4] This difference may be due to intrinsic variation in folate contents between the cultivars analyzed or the accuracy of the methods used for folate quantitation. Additionally, nutritional tables from USDA and other institutes do not specify the papaya variety used for the determinations.[4,5] On the other hand, the majority of the folate values reported in food tables are obtained using the microbiological assay, which is based on a folate-dependent bacterial growth; this assay can blindly underestimate folate contents if the deglutamylation step for polyglutamylated folates is not complete during the

sample processing. Conversely, the chromatographic method used in this work is able to detect polyglutamylated folate peaks (Figure 2A), which were not observed in the deglutamylated samples (Figure 2B), demonstrating complete hydrolysis that allowed for a more accurate quantitation of folate classes grouped as monoglutamyl forms. In addition, the electrochemical detector used is more sensitive than the bacterial assay.[18] Other works have reported changes in folate levels during fruit ripening: in tomato fruit, two prior studies found differences in folate accumulation trends during ripening among several tomato varieties; the majority showed a decrease when folate contents were compared in mature green and fully ripened stages.[19,20] However, in a separate study, tomato fruit from the Microtom cultivar did not show significant differences in folate contents during ripening.[21] Furthermore, when folates were quantified in strawberries during three consecutive harvesting years, folate levels changed in opposite trends during ripening depending on the year of harvest, and no evident pattern could be established.[22] All of these data suggest that folate pools in fruits could be sensitive to abiotic conditions when the fruit is ripened on the plant and to postharvest environments when it is ripened off the plant; therefore, we cannot establish or predict folate accumulation trends during fruit ripening. Further work that focuses on finding the abiotic variables that affect folate accumulation in fruit during development and postharvest handling would help to consider these variables to maintain or even enhance folate pools in fruits. In the case of papaya fruit, ripening at room temperature off the plant, with no ethylene addition, led to a slight increase in folate accumulation that represents 24.5% of the adult recommended dietary allowance (RDA, 400 μg/day) if one fully ripened portion of 1 cup (145 g) is consumed (Table 1).

Papaya fruit accumulates folates in all stages mainly as THF and 5-CH_3-THF (Table 1), the latter being usually the predominant folate found in some other fruits.[23,24] THF (which in our analysis comprises 5,10-methylene-THF, 5,10-CH_2-THF, at the acidic pH of the mobile phase) has also been found to be predominant in soybean.[25] On the other hand, 5,10-methenyl-THF (5,10-CH=THF, which includes 10-formyl-THF, 10-CHO-THF, as well) and 5-CHO-THF were part of the fruit folate pool in minor amounts (7 and 2%, respectively, of total folates in fully mature fruit; Table 1). 5,10-CH=THF increased significantly when the fruit was fully ripened, whereas 5-CHO-THF levels decreased during developmental stages 0.5 and 0.8, maintaining its levels with no change during all ripening stages.

Characterization of the Polyglutamylation Profiles in *C. papaya* cv. Maradol. Folates exist in nature mainly with a polyglutamyl tail that helps to retain folates within tissues and subcellular compartments, because monoglutamylated folates are the main transported forms.[9,26] The polyglutamyl tails reported for plants usually comprise up to eight glutamyl residues (Glu8);[27] however, there is a single study that observed tentatively up to 14 Glu residues in spinach,[28] but no further characterization was reported afterward. Only when folates were engineered in *Lactococcus lactis* by overexpressing the folylpolyglutamate synthetase (FPGS) was a longer glutamyl chain length reported, up to 12 glutamyl residues attached to 5,10-CH=THF.[29] In this study, unexpectedly, we found very long polyglutamyl chains linked to 5-CH_3-THF in papaya fruit (Figure 2A). Our peak assignment was based on previous works,[17,30] which demonstrated that in a reverse-phase ion pair HPLC column, polyglutamylated folates elute sequentially as the number of glutamate residues within the tail increase. On this basis and the fact that each folate derivative presents a distinct response pattern in each of the four channels of the electrochemical detector (Figure 2B),[30] we counted in our chromatograms the elution of at least 17 peaks that corresponded to the 5-CH_3-THF pattern in papaya fruit extracts (Figure 2A). Moreover, when the same folate extracts were deglutamylated with conjugase treatment, all of the peaks that were considered to be 5-CH_3-THF polyglutamylated species disappeared from the chromatogram, and the peak areas of the monoglutamyl form increased accordingly (Figure 2A,B).

To further confirm peak assignments as 5-CH_3-THF polyglutamyl chains, in an additional study, we compared the retention times and response profile in each detector channel of the first 5-CH_3-THF polyglutamyl species (2−7 residues) to those of 5-CH_3-THF tetra- and heptaglutamyl standards (Figure S1, Supporting Information) and to human erythrocyte and tomato folate extracts for which polyglutamyl profiles are available in the literature.[21,31] Those two matrices were selected for comparison purposes because neither other references nor standards of longer polyglutamylated folates are available. One concern for the true assignment of a peak as 5-CH_3-THF polyglutamyl was the possible coelution with other folate forms. In particular, this applies to THF polyglutamyl forms, because they present similar response in the channels and elute close to 5-CH_3-THF (Figure 2B).

To provide more evidence for the 5-CH_3-THF polyglutamylated form identity, we analyzed the same papaya fruit folate extract in another HPLC system coupled to a fluorescence detector (Figure 2C). This additional methodology was selected because 5-CH_3-THF has higher fluorescence emission at 358 nm when excited at 285 nm at the acidic mobile phase at pH of 2.5, whereas other folate derivatives, such as THF, have just 13% of 5-CH_3-THF fluorescence response, and others, such as 5,10-CH=THF, do not fluoresce under these conditions.[18] Results indicated that 5-CH_3-THF polyglutamyl profile was consistent regardless of the detector used (Figure 2A,C) and the polyglutamyl profile disappeared after deglutamylation, leaving just THF and 5-CH_3-THF signals for the fluorescence detector (Figure 2D). These results support the assigned identity of the polyglutamylated multiple peaks observed as derivatives of the 5-CH_3-THF folate form. In the present work, we quantified polyglutamyl species as 5-CH_3-THF monoglutamylated equivalents because calibration curves constructed with the tetraglutamylated standard showed a slight decrease in the response of our electrochemical cell (85% of the monoglutamyl signal), although other authors did not report significant changes when 5-CH_3-THF pentaglutamyl standard was used.[30] Quantification of polyglutamylated 5-CH_3-THF signals in monoglutamyl equivalents resulted in a total polyglutamylated concentration that was very close to the total concentration of the deglutamylated samples (Table 1; Table S2, Supporting Information); therefore the selected quantification procedure seems to be appropriate to avoid overestimation.

To confirm chemical identities of the tentative papaya polyglutamyl folate profiles, reported here, further work using peak isolation and mass spectrometry detection would be needed. Nevertheless, for the purpose of discussion in this paper, we are tentatively assigning chain length numbers to peaks with the same electrochemical and fluorescence response as they elute. Overall, we consider that the group of evidence

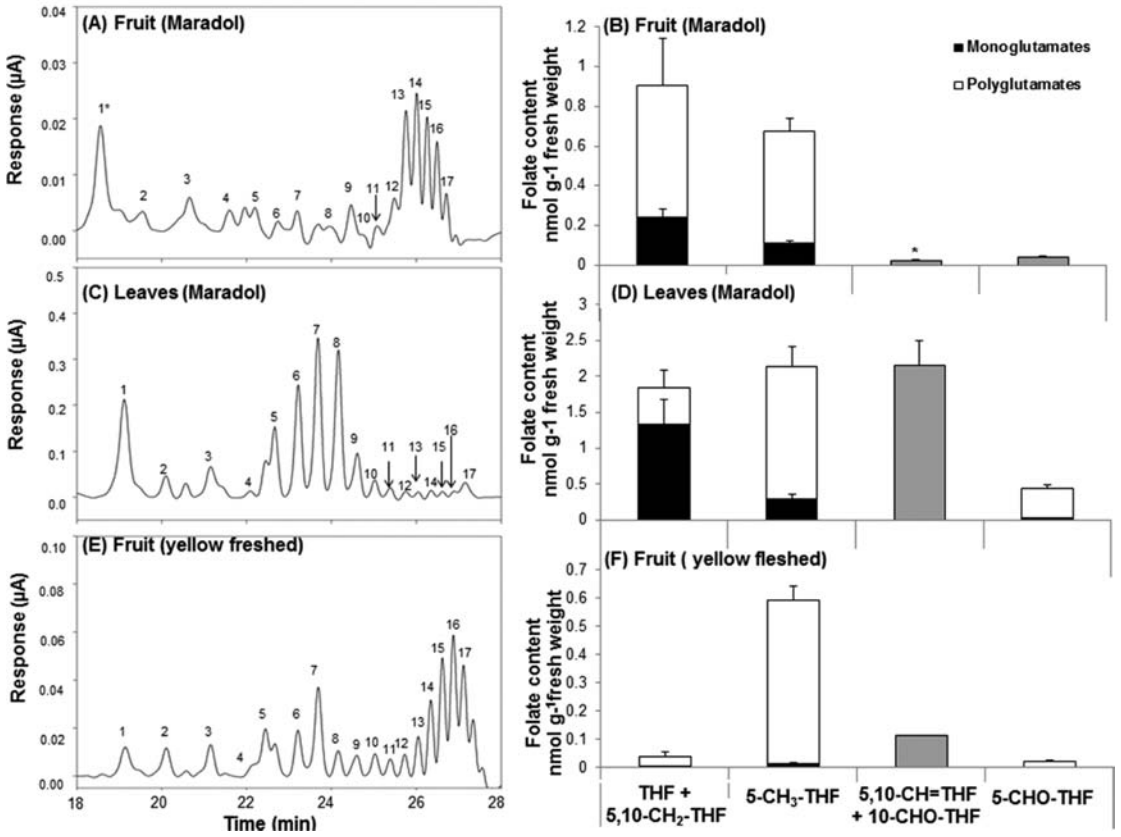

Figure 3. Folate polyglutamylation profile and accumulation in papaya fruit and leaves: HPLC chromatograms showing polyglutamylation profiles of 5-CH_3-THF in papaya fruit and leaves (A, C, E); polyglutamylation extent of folate classes (B, D, F). Polyglutamyl contribution in each folate class was calculated by subtracting monoglutamyl values obtained from polyglutamylation profile analysis (without incubation with conjugase) from those deglutamylated. Data are the mean and SE of three independent determinations. (*) In the Maradol cultivar, 5-CHO-THF polyglutamyl contribution (gray bar) could not be assessed due to peak coelution. 5,10-CH=THF monoglutamylated could not be quantified in nondeglutamylated profiles, due also to coelution (gray bars). THF, tetrahydrofolate.

presented here indicates that papaya fruit produces and accumulates 5-CH_3-THF predominantly with a very high polyglutamyl chain that has not been reported before for a nonmodified organism.

To establish whether this very high level of polyglutamylation was specific to papaya fruit or specific to the Maradol variety, we analyzed folate profiles in leaves from the Maradol tree and in fruit from a yellow-fleshed papaya (at ripening stage 3). We found very high polyglutamylation levels in both samples (Figure 3C,E). In leaves, which accumulate 3.9 times more folates than fruit mesocarp, as has been observed in other plants (green leaves accumulate more folates than sink tissues[32]), we were also able to detect up to 17 peaks of 5-CH_3-THF (Figure 3C). Fruit mesocarp from the yellow papaya also presented a high folate polyglutamylation profile, very similar to that observed for the Maradol cultivar (Figure 3E); however, we found higher total folate concentrations in Maradol fruit compared with yellow-fleshed papaya at the same ripening stage (Figure 3B,F, respectively). The differences in folate contents found among these samples can be expected; the yellow-fleshed papaya bought at the market might have had different conditions of growth, harvest, and postharvest handling that are unknown to us; in addition, this papaya fruit accumulated significantly less THF than the Maradol cultivar. Whereas all of the samples had very high polyglutamylated folate species, in leaves, the predominant 5-CH_3-THF forms contained mainly five to eight residues, which comprised 63.5% of the pool (Figure 3C), and THF was primarily monoglutamylated (Figure 3D). On the other hand, both fruit samples had the majority of the THF pool polyglutamylated (Figure 3B,F), and the main 5-CH_3-THF forms were also very highly polyglutamylated (Figure 3A,E). These data demonstrate that the high level of polyglutamylation of 5-CH_3-THF found in papaya is not exclusive for fruit tissues or the Maradol variety. They show additionally that the polyglutamyl profile has variations depending on the tissue, which could be due to their metabolic differences.

The polyglutamylation extent of folates depends on the activity of two enzymes that add or hydrolyze glutamate residues, folylpolyglutamate synthase (FPGS) and γ-glutamyl hydrolase (GGH), respectively. These two enzymes are present in plants, animals, and microorganisms;[24,33,34] in plants, there is more than one isoform for both enzymes, and they are targeted to different subcellular compartments. GGHs have been localized in vacuoles of pea leaves,[16] whereas in *Arabidopsis*, FPGS isoforms b, c, and d have been found in plastids, mitochondria, and cytosol, respectively.[35] Nothing is known about *C. papaya* homologues for these two enzymes; their activities and regulation might generate the folate profiles that were detected in this plant. On the other hand, the functional role of folate polyglutamylation in plant metabolism and development has just begun to be explored in recent years. Lack of FPGS isoforms results in fewer polyglutamylated folate species, causing several detrimental phenotypes in *Arabidopsis*

plants,[36,37] whereas lack and overexpression of GGH in *Arabidopsis* and tomato fruit resulted in an increase and decrease of the folate pool, respectively.[38] Furthermore, the low polyglutamylated folate status found in tomato fruit had a negative effect on fruit size and seed set.[38] All of these observations raise questions about the metabolic and developmental effect that this high polyglutamylation profile causes in papaya tissues, if any. Moreover, nothing is known about the degradation kinetics of these folate derivatives during food processing and storage and their effect on folate bioavailability. With regard to folate bioavailability, the differences in bioavailability and bioequivalence of folates mono- and polyglutamylated have not been completely established. Studies in humans have shown less bioavailability of folic acid hexa- and heptaglutamylated compared with that of the monoglutamyl form; however, the bioequivalence calculated for both forms was not significantly different.[39,40] These previous works have used polyglutamylated folic acid, which does not exist naturally and may not have the same behavior as the reduced polyglutamyl forms. Papaya plant is able to produce fair amounts of natural polyglutamylated forms of the main folate class found in foods; which may facilitate future bioavailability studies. Because papaya is considered to be a good folate source, it is appealing to investigate the bioavailability of folates from this food plant.

Changes in Polyglutamylation Profiles of 5-CH_3-THF during Fruit Development and Ripening. The extent of polyglutamylation of the 5-CH_3-THF was followed during three phases of fruit development and five ripening stages (Figure 4; Table S2, Supporting Information). The accumulation of very long polyglutamylated (peaks 11−17) folate forms was observed from early stages of development (fruit developmental stage 0.3, Figure 4C); 50% of the total 5-CH_3-THF had a tail of >11 glutamates (Glu). On the other hand, 25% of the 5-CH_3-THF pool was composed by mono- and diglutamate molecules (Figure 4A). 5-CH_3-THF monoglutamyl levels diminished through the rest of the development; however, at the onset of ripening (stage 1), they rapidly accumulated until the third stage, representing almost one-fourth of the 5-CH_3-THF pool. The last two ripening stages of the papaya fruit had very low contributions from the short polyglutamyl classes, and >72% of the 5-CH_3-THF had polyglutamyl tails above Glu7. During development and ripening, there are some polyglutamyl forms that did not change significantly and had low contributions to the pool (Figure 4A,B; Glu4, Glu5, Glu9, and Glu10). Conversely, the polyglutamylated forms that varied the most were Glu8, Glu11, and Glu17. There was a clear trend of accumulation of the Glu17 forms, which correlates with the decrease of the lower immediate polyglutamyl classes (Figure 4C) during stages 4 and 5. Additionally, there was an evident change in the 5-CH_3-THF polyglutamyl profile that occurred at the end of fruit development and at the onset of ripening; at stage 1, almost 30% of the 5-CH_3-THF was monoglutamylated, whereas the previous stage, 0.8, had just 6.2% as monoglutamyl form (Figure 4A). The total 5-CH_3-THF pool also increased 27% at the onset of ripening (Table 1); thus, this increase in the total pool was mainly due to the accumulation of 5-CH_3-THF monoglutamylated. After this steep change, the monoglutamyl form maintained similar levels until stages 4 and 5, when it declined to the levels found during fruit development. Interestingly, the exact opposite trend can be observed for the very high polyglutamyl forms (Figure 4C). Very little is

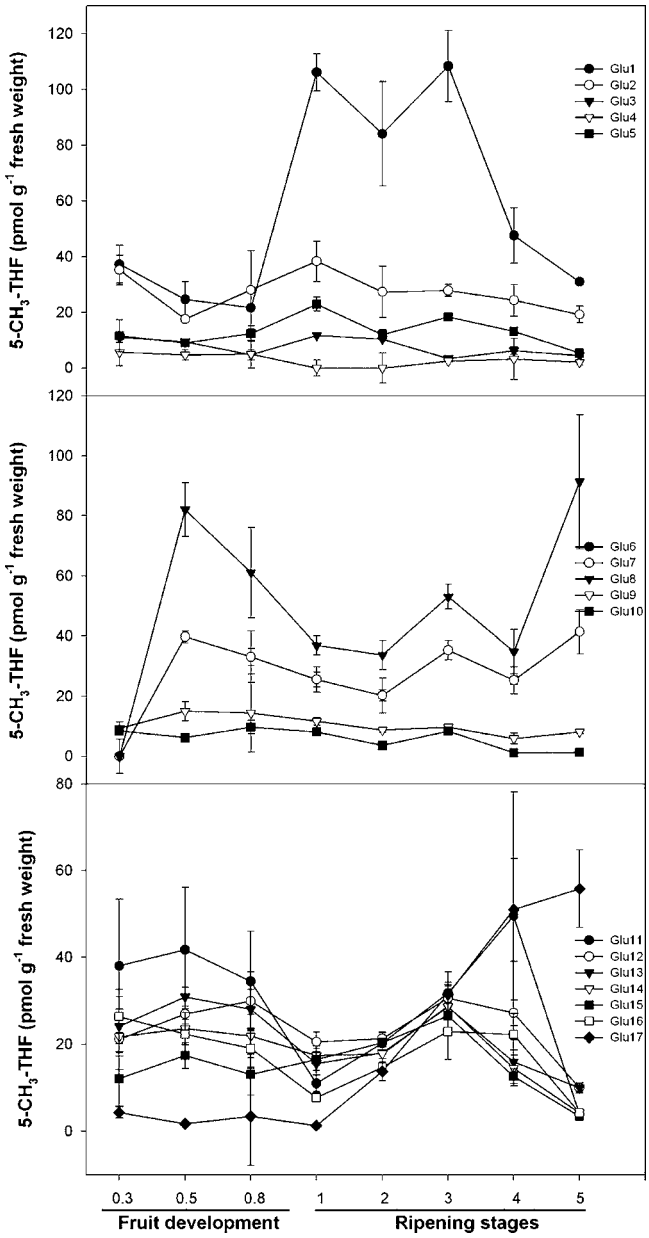

Figure 4. Changes in polyglutamylation extent of 5-CH_3-THF during fruit development and ripening: contents of low polyglutamyl tail (1−5 glutamate residues) (A), high polyglutamyl tail (6−10 glutamate residues) (B), and very high polyglutamyl tail (11−17 glutamate residues) (C). Glu, glutamate. All data points are the average ± SE of three independent determinations. All polyglutamyl species of 5-CH_3-THF were quantified relative to monoglutamylated 5-CH_3-THF.

known about the regulation of folate metabolism in ripening fruits and the folate role during this process; papaya is a climacteric fruit that presents a peak of ethylene at the onset of ripening.[41] Ethylene is synthesized from methionine via *S*-adenosylmethionine, which can be metabolized to 1-aminocyclopropane-1-carboxylic acid (ACC), its immediate precursor. Methionine is formed through methylation of homocysteine by methionine synthase, which utilizes 5-CH_3-THF as a methyl donor;[42] this reaction represents the major metabolic flux for folates in plants.[24] We are tempted to hypothesize that the onset of ripening would represent an increase of the folate flux toward 5-CH_3-THF, which at high fluxes could not be immediately polyglutamylated by FPGS. Previous work

supports this speculation: when folates were engineered in tomato, the high metabolic flux that resulted from the enhanced folate biosynthesis had as a consequence a very high increase in 5-CH_3-THF monoglutamyl compared with that observed in the main folate class in wild-type tomatoes (5-CH_3-THF hexaglutamyl).[21] Moreover, these engineered tomatoes had >2-fold increase in the expression of the ACC oxidase gene, which codes for the last enzyme in ethylene biosynthesis.[43] These observations hint of a folate function during fruit ripening possibly linked to ethylene biosynthesis. Further work will be needed to shed some light into the role of folates in fruit development and ripening processes.

Data presented in this work showed that there are singular folate profiles that are still to be discovered in nonmodel plants. These unusual profiles raise questions about the role of folate polyglutamylation in plants. Knowledge generated from other plant species will contribute to the understanding of the regulation of folate metabolism, which is limited nowadays. This study also confirms that fully ripe papaya represents a good folate source, and it raises questions about the bioavailability of folates from this food matrix.

■ ASSOCIATED CONTENT

Supporting Information

Chromatographic profiles of papaya fruit folate extracts with added polyglutamylated folate standards, physicochemical properties of papaya fruit during expansion and ripening, and polyglutamylated 5-CH_3-THF content of papaya fruit during development and ripening. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS USED

ACC, 1-aminocyclopropane-1-carboxylic acid; 5-CH_3-THF, 5-methyltetrahydrofolate; 5-CHO-THF, 5-formyltetrahydrofolate; 5,10-CH=THF, 5,10-methenyltetrahydrofolate; FPGS, folylpolyglutamate synthase; GGH, γ-glutamyl hydrolase; Glu, glutamyl residues; PABA, *p*-aminobenzoate; RDA, Recommended Dietary Allowance; THF, tetrahydrofolate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CHES, *N*-cyclohexyl-2-aminoethanesulfonic acid

■ REFERENCES

(1) Gayosso-García, L. E.; Yahia, E. M.; González-Aguilar, G. A. Identification and quantification of phenols, carotenoids, and vitamin C from papaya (*Carica papaya* L., cv. Maradol) fruit determined by HPLC-DAD-MS/MS-ESI. *Food Res. Int.* **2011**, 1−8.

(2) de Souza, L. M.; Ferreira, K. S.; Paes Chaves, J. B.; Teixeira, S. L. L-Ascorbic acid, β-carotene and lycopene content in papaya fruits (*Carica papaya*) with or without physiological skin freckles. *Sci. Agric.* **2008**, *65*, 246−250.

(3) Chandrika, U. G.; Jansz, E. R.; Wickramasinghe, S. M.; Warnasuriya, N. D. Bioconversion of pro-vitamin A carotenoids and antioxidant activity of *Carica papaya* fruits. *J. Natl. Sci. Found. Sri Lanka* **2010**, *31*, 437−444.

(4) U.S. Department of Agriculture, Agricultural and Research Service. *USDA National Nutrient Database for Standard Reference, release 24*, Nutrient Data Laboratory Home Page, 2011; http://www.ars.usda.gov/ba/bhnrc/ndl.

(5) ANSES. Ciqual French food composition table version 2012. http://www.anses.fr/TableCIQUAL/index.htm (accessed Nov 8, 2012).

(6) Hanson, A. D.; Roje, S. One-carbon metabolism in higher plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **2001**, *52*, 119−137.

(7) Lucock, M. Folic acid: nutritional biochemistry, molecular biology, and role in disease processes. *Mol. Genet. Metab.* **2000**, *71*, 121−138.

(8) Scott, J.; Rebeille, F.; Fletcher, J. Folic acid and folates: the feasibility for nutritional enhancement in plant foods. *J. Sci. Food Agric.* **2000**, *80*, 795−824.

(9) Tibbetts, A. S.; Appling, D. R. Compartmentalization of mammalian folate-mediated one-carbon metabolism. *Annu. Rev. Nutr.* **2010**, *30*, 57−81.

(10) Wei, M. M.; Gregory, J. F. Organic acids in selected foods inhibit intestinal brush border pteroylpolyglutamate hydrolase in vitro: potential mechanism affecting the bioavailability of dietary polyglutamyl folate. *J. Agric. Food Chem.* **1998**, *46*, 211−219.

(11) FAOSTAT. Food and agricultural commodities production; http://faostat.fao.org/DesktopDefault.aspx?PageID=339&lang=en (accessed 2012).

(12) SAGARPA. Servicio de Información Agroalimentaria y Pesquera (SIAP): Cierre de la producción agrícola por cultivo; www.siap.gob.mx (accessed 2012)

(13) Blas, A. L.; Ming, R.; Liu, Z.; Veatch, O. J.; Paull, R. E.; Moore, P. H.; Yu, Q. Cloning of the papaya chromoplast-specific lycopene beta-cyclase, CpCYC-b, controlling fruit flesh color reveals conserved microsynteny and a recombination hot spot. *Plant Physiol.* **2010**, *152*, 2013−2022.

(14) Association of Official Analytical Chemists (AOAC). *Official Methods of Analysis of the Association of Official Analytical Chemists*, 16th ed.; AOAC: Washington, DC, 1995.

(15) Díaz de la Garza, R.; Quinlivan, E. P.; Klaus, S. M.; Basset, G. J.; Gregory, J. F.; Hanson, A. D. Folate biofortification in tomatoes by engineering the pteridine branch of folate synthesis. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 13720−13725.

(16) Orsomando, G.; Díaz de la Garza, R.; Green, B. J.; Peng, M.; Rea, P. A.; Ryan, T. J.; Gregory, J. F.; Hanson, A. D. Plant-glutamyl hydrolases and folate polyglutamates: characterization, compartmentation, and co-occurrence in vacuoles. *J. Biol. Chem.* **2005**, *280*, 28877−28884.

(17) Selhub, J. Determination of tissue folate composition by affinity chromatographyfollowed by high pressure ion pair chromatography. *Anal. Biochem.* **1989**, *182*, 84−93.

(18) Quinlivan, E. P.; Hanson, A. D.; Gregory, J. F. The analysis of folate and its metabolic precursors in biological samples. *Anal. Biochem.* **2006**, *348*, 163−184.

(19) Periago, M. J.; García-Alonso, J.; Jacob, K.; Olivares, B.; Bernal, J.; Iniesta, M. D.; Martínez, C.; Ros, G. Bioactive compounds, folates and antioxidant properties of tomatoes (*Lycopersicum esculentum*) during vine ripening. *Int. J. Food Sci. Nutr.* **2008**, 1−15.

(20) Iniesta, M. D.; Pérez-Conesa, D.; García-Alonso, J.; Ros, G.; Periago, M. J. Folate content in tomato (*Lycopersicon esculentum*). Influence of cultivar, ripeness, year of harvest, and pasteurization and storage temperatures. *J. Agric. Food Chem.* **2009**, *57*, 4739−4745.
(21) Diaz de la Garza, R. I.; Gregory, J. F. I.; Hanson, A. D. Folate biofortification of tomato fruit. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 4218−4222.
(22) Strålsjö, L. M.; Witthöft, C. M.; Sjöholm, I. M.; Jägerstad, M. I. Folate content in strawberries (*Fragaria* × *ananassa*): effects of cultivar, ripeness, year of harvest, storage, and commercial processing. *J. Agric. Food Chem.* **2003**, *51*, 128−133.
(23) Konings, E. J.; Roomans, H. H.; Dorant, E.; Goldbohm, R. A.; Saris, W. H.; van den Brandt, P. A. Folate intake of the Dutch population according to newly established liquid chromatography data for foods. *Am. J. Clin. Nutr.* **2001**, *73*, 765−776.
(24) Hanson, A. D.; Gregory, J. F. Folate biosynthesis, turnover, and transport in plants. *Annu. Rev. Plant Biol.* **2011**, *62*, 105−125.
(25) Rychlik, M.; Englert, K.; Kapfer, S.; Kirchhoff, E. Folate contents of legumes determined by optimized enzyme treatment and stable isotope dilution assays. *J. Food Compos. Anal.* **2007**, *20*, 411−419.
(26) Li-Li, Z.; Ying, L.; Song, L.; Cossins, E. A. The polyglutamate nature of plant folates. *Phytochemistry* **1992**, *31*, 2277−2282.
(27) Cossins, E. A. The fascinating world of folate and one-carbon metabolism. *Can. J. Bot.* **2000**, *78*, 691−708.
(28) Garratt, L. C.; Ortori, C. A.; Tucker, G. A.; Sablitzky, F.; Bennett, M. J.; Barrett, D. A. Comprehensive metabolic profiling of mono- and polyglutamated folates and their precursors in plant and animal tissue using liquid chromatography/negative ion electrospray ionisation tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2005**, *19*, 2390−2398.
(29) Sybesma, W.; Van Den Born, E.; Starrenburg, M.; Mierau, I.; Kleerebezem, M.; de Vos, W. M.; Hugenholtz, J. Controlled modulation of folate polyglutamyl tail length by metabolic engineering of *Lactococcus lactis*. *Appl. Environ. Microbiol.* **2003**, *69*, 7101−7107.
(30) Bagley, P. J.; Selhub, J. Analysis of folate form distribution by affinity followed by reversed-phase chromatography with electrochemical detection. *Clin. Chem.* **2000**, *46*, 404−411.
(31) Pfeiffer, C. M.; Gregory, J. Enzymatic deconjugation of erythrocyte polyglutamyl folates during preparation for folate assay: investigation with reversed-phase liquid chromatography. *Clin. Chem.* **1996**, *42*, 1847−1854.
(32) Sahr, T.; Ravanel, S.; Rébeillé, F. Tetrahydrofolate biosynthesis and distribution in higher plants. *Biochem. Soc. Trans.* **2005**, *33*, 758−762.
(33) Moran, R. G. Characterization of the function of mammalian folylpolyglutamate synthetase (FPGS). *Adv. Exp. Med. Biol.* **1983**, *163*, 327−339.
(34) El Fadili, A.; Kündig, C.; Ouellette, M. Characterization of the folylpolyglutamate synthetase gene and polyglutamylation of folates in the protozoan parasite *Leishmania*. *Mol. Biochem. Parasitol.* **2002**, *124*, 63−71.
(35) Ravanel, S.; Cherest, H.; Jabrin, S.; Grunwald, D.; Surdin-Kerjan, Y.; Douce, R.; Rebeille, F. Tetrahydrofolate biosynthesis in plants: molecular and functional characterization of dihydrofolate synthetase and three isoforms of folylpolyglutamate synthetase in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 15360−15365.
(36) Srivastava, A. C.; Ramos-Parra, P. A.; Bedair, M.; Robledo-Hernandez, A. L.; Tang, Y.; Sumner, L. W.; Diaz de la Garza, R. I.; Blancaflor, E. B. The folylpolyglutamate synthetase plastidial isoform is required for postembryonic root development in *Arabidopsis*. *Plant Physiol.* **2011**, *155*, 1237−1251.
(37) Mehrshahi, P.; Gonzalez-Jorge, S.; Akhtar, T. A.; Ward, J. L.; Santoyo-Castelazo, A.; Marcus, S. E.; Lara-Núñez, A.; Ravanel, S.; Hawkins, N. D.; Beale, M. H.; Barrett, D. A.; Knox, P. J.; Gregory, J. F.; Hanson, A. D.; Bennett, M. J.; Dellapenna, D. Functional analysis of folate polyglutamylation and its essential role in plant metabolism and development. *Plant J.* **2010**, *64*, 267−279.
(38) Akhtar, T. A.; Orsomando, G.; Mehrshahi, P.; Lara-Núñez, A.; Bennett, M. J.; Gregory, J. F.; Hanson, A. D. A central role for gamma-glutamyl hydrolases in plant folate homeostasis. *Plant J.* **2010**, *64*, 256−266.
(39) Melse-Boonstra, A.; West, C. E.; Katan, M. B.; Kok, F. J.; Verhoef, P. Bioavailability of heptaglutamyl relative to monoglutamyl folic acid in healthy adults. *Am. J. Clin. Nutr.* **2004**, *79*, 424−429.
(40) Melse-Boonstra, A.; Verhoef, P.; West, C. E.; van Rhijn, J. A.; van Breemen, R. B.; Lasaroms, J. J.; Garbis, S. D.; Katan, M. B.; Kok, F. J. A dual-isotope-labeling method of studying the bioavailability of hexaglutamyl folic acid relative to that of monoglutamyl folic acid in humans by using multiple orally administered low doses. *Am. J. Clin. Nutr.* **2006**, *84*, 1128−1133.
(41) Fabi, J. P.; Cordenunsi, B. R.; de Mattos Barreto, G. P.; Mercadante, A. Z.; Lajolo, F. M.; Oliveira do Nascimento, J. R. Papaya fruit ripening: response to ethylene and 1-methylcyclopropene (1-MCP). *J. Agric. Food Chem.* **2007**, *55*, 6118−6123.
(42) Ravanel, S. Methionine metabolism in plants: Chloroplasts are autonomous for de novo methionine synthesis and can import *S*-adenosylmethionine from the cytosol. *J. Biol. Chem.* **2004**, *279*, 22548−22557.
(43) Waller, J. C.; Akhtar, T. A.; Lara-Núñez, A.; Gregory, J. F.; McQuinn, R. P.; Giovannoni, J. J.; Hanson, A. D. Developmental and feedforward control of the expression of folate biosynthesis genes in tomato fruit. *Mol. Plant* **2010**, *3*, 66−77.